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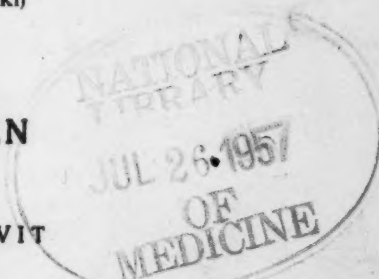
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**EFFECT OF ADRENOCORTICAL HORMONES ON  
HYDROXYPROLINE CONCENTRATION OF HEART  
AND SKELETAL MUSCLE**

**AN EXPERIMENTAL INVESTIGATION WITH MICE**

BY

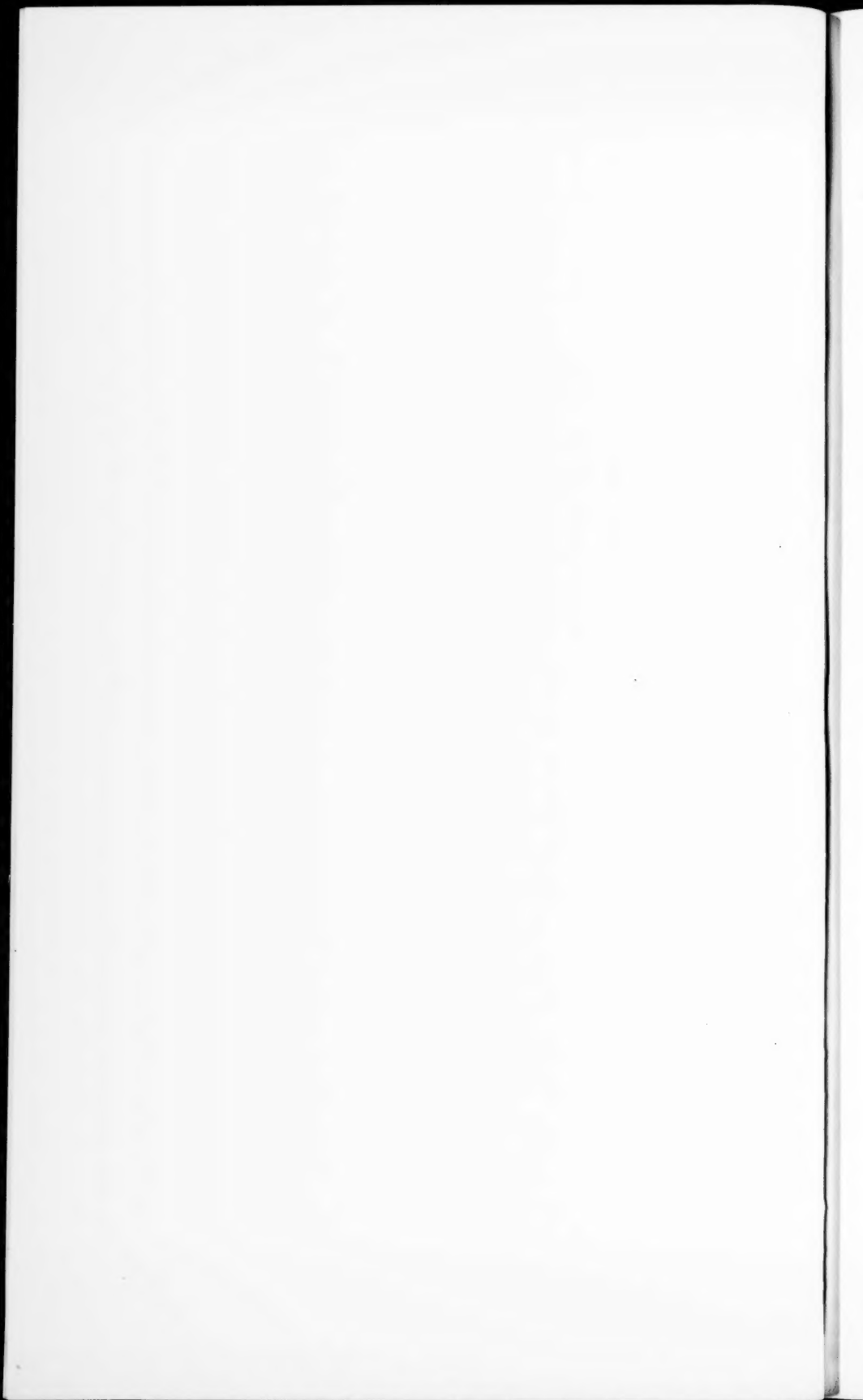
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HELSINKI 1957



## PREFACE

It is a great pleasure to express my deep gratitude to Docent M. J. Karvonen, M.D., Ph. D., Chief of the Physiological Department of the Institute of Occupational Health, Helsinki, for suggesting this problem, for laboratory facilities, and for his untiring interest and valuable support and encouragement in every phase of the work.

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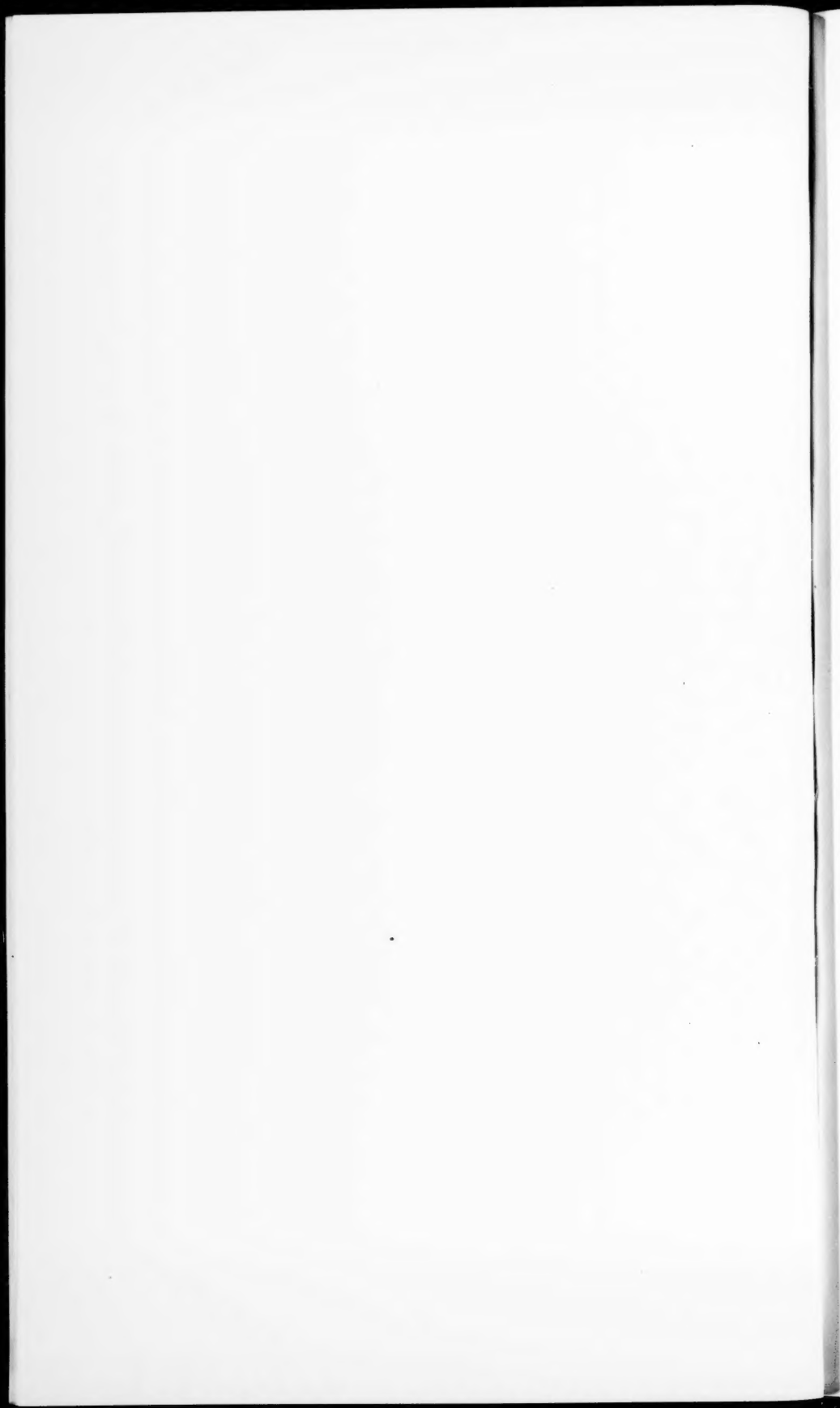
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Helsinki, January 1957.

*Ora Friberg*



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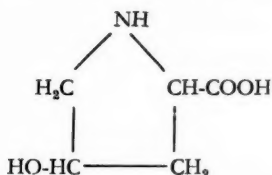
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## INTRODUCTION

*Hydroxyproline* was isolated in 1902 by Fischer from an acid hydrolysate of gelatin. By means of phosphorus and hydriodic acid he reduced the hydroxyl group under conditions which yielded racemic proline. Leusch (1905) synthesized the compound, and with Brewster (1913) separated the proper isomer from the reaction mixture and showed that it was identical with the substance isolated from proteins.

Later, Leusch and Bormann (1919) prepared the three remaining optical isomers. From the fact that no  $\gamma$ -lactone could be formed from acetyl-L-hydroxyproline, Kaneko (1940) deduced that the carboxyl and hydroxyl radicals are on opposite sides of the molecule. Thus, the molecular structure of hydroxyproline, 4-hydroxy-2-pyrrolidinecarboxylic acid, is as follows:



The collagen group of proteins is characterized by the prominence of proline and hydroxyproline, and the paucity of aromatic residues. In particular, the large amount of hydroxyproline is unique, and also important for the characterization of collagen as a class, and for its estimation. Hydroxyproline is apparently present in significant amounts in no other protein except elastin, which contains 1.5 to 2.3 per cent of hydroxyproline (Neuman and Logan 1950a). The hydroxyproline content of mammalian collagen is

stated to be rather constant, 13 to 14 per cent, but fish-skin collagen, for instance, contains only 6 to 9 per cent of hydroxyproline. In view of these observations and the fact that the hydrothermal stability of collagens from mammalian and teleost sources differ significantly, Gustavson (1955) suggested that the function of hydroxyproline possibly is to stabilize collagen. This agrees with the recent work of Burton *et al.* (1955) who suggest that hydroxyproline residues may be of particular importance in aiding the cohesion of collagen fibrils. They also claim that there is possibly a close association of hydroxyproline and polysaccharide in collagen.

According to Bear (1952) collagen fibres have a high concentration of proline and hydroxyproline. The hydroxyproline content of most tissues probably depends on their collagen content. Thus, many investigations on collagen content of different organs have been worked out by using the photometric method of Neuman and Logan (1950b), for instance by Harkness (1952), Harkness and Harkness (1954a, b, 1955a, b), Harkness *et al.* (1954), Aterman (1954), Roberts *et al.* (1951), Bowes *et al.* (1953).

#### CONNECTIVE TISSUE OF HEART AND SKELETAL MUSCLE

The following short general survey is based on the presentations by Maximow and Bloom (1952), Angevine (1950), Ragan (1952), and Bear (1952).

The parallel muscle fibres which form the muscle are held together by loose connective tissue. To this tissue of adult organism four definite functions have been ascribed: (a) support, (b) transport, (c) storage, and (d) repair (healing) and protection (antibody formation). In the loose connective tissue there are cellular and intercellular elements between which there are spaces filled with amorphous ground substance. In the different types of connective tissues there are several fibrillar constituents, collagenous, elastic, and reticular fibres.

*Collagenous fibres* are formed of bundles of small fibrils which run in a wavy course and are very flexible and resistant to traction. They consist of an albuminoid, called collagen. This material can be converted to gelatin by boiling in aqueous solutions. In weak acids or alkalies, collagenous fibres swell; in strong acids or alkalies they are likely to dissolve or to be destroyed. Besides of these physical and chemical characteristics they may be histologically recognized by their resistance to degradation by enzymes, by a pronounced shrinkage in length at elevated temperatures or in certain electrolyte solution, by an immunological inertness, and by rather non-specific affinities for some dyes, such as acic fuchsin or aniline blue. Collagen fibrils are composed of thinner fundamental units, called protofibrils, as shown by X-ray diffraction. The protofibrils are parallel and axially arranged so that corresponding particulars of chemical structure more or less match transverse to the fibril axis. Each protofibril carries a periodic pattern of chemical content along it.

Chemically, collagens from various sources have small differences, but a common characteristic in the amino acid composition is the abundance of glycine and hydroxyproline.

While collagen fibres are found within the connective tissue, *reticular fibres* can be found wherever connective tissue forms a boundary. Thus, the sarcolemma around an individual skeletal muscle fibre is composed of reticular fibres, and so is the thin membrane surrounding the endothelial cells of capillaries and larger vessels. The supporting stroma of many organs is composed of fine reticular fibres which characteristically branch and anastomose with each other to form a network, called *reticulum*. The reticular and collagen fibrils are in direct continuity with each others. E.g. the reticular fibres of sarcolemma gradually change to the collagen fibres of the tendon, thus transmitting the contraction of the muscle. The reticular fibres consist of a protein called reticulin.

Ever since 1888 when Mall isolated reticulin from lymphonodes and failed to produce gelatin when boiling reticulin, there has been dispute as to whether the composition of collagen and reticulin is similar or not. Histologically, collagen and reticular fibrils differ from each others in their affinity to stains. Reticular fibres are argyrophilic when appropriately treated with silver, and they stain poorly with fuchsin. However, it is generally agreed that collagen fibres in their early stages of development conform in all respects to the histological definition of reticulin. Kramer and Little (1953) have found a close relation between collagen and reticulin in their reactions to enzymes, in the electron microscope appearance of the fibrillar components, and in the X-ray diffraction pattern; both of them give the reactions of proteins and both contain remarkably few aromatic residues, have high hydroxyproline content, and contain only traces of sulphur and phosphorus.

The *elastic fibres* are much less numerous than collagenous and reticular fibres. Elastic fibres are single, they branch and anastomose freely. They are highly refractile and elastic. They consist of a protein, called elastin, which is highly resistant even to strong acids and alkalis, and also resists boiling. The amino acid composition of elastin is different from that of collagen. Thus, elastin contains only 1.5 per cent hydroxyproline (Neuman and Logan). If the tissue contains plenty of elastic fibres it has a yellow hue. In the contrary, collagenous fibres yield white colour.

## HORMONAL REGULATION OF FIBROGENESIS

The importance of the mesenchymal tissues in disease and defence mechanisms has aroused considerable interest into the influence of various hormones upon the formation and structure of these tissues. The work of Hench *et al.* (1950) on adrenocortical hormones and rheumatoid arthritis has stimulated the study of the effects of corticoids on the connective tissue. It is widely recognized that during induced hyperadrenalism, fibroplasia is delayed (Blunt *et al.* 1950, Creditor *et al.* 1950, Ragan *et al.* 1949, Spain *et al.* 1950). The inhibitory effect is apparently directed upon cell preceding the fibroblast in development. Once fibroblast has attained its maturity, collagen and ground substances are deposited. In scurvy, on the contrary, the inhibitory effect is on the function of the fibroblast after its appearance (Ragan 1952). In the experiments of Taubenhaus and Amromin (1950) hypophysectomized animals failed to develop around a turpentine abscess the

amount of granulation tissue observed in normal animals. The anterior pituitary stimulated fibroplastic proliferation through its growth hormone. In intact animals growth hormone also produced stimulation; however, if injected in large doses it inhibited such tissues. Testosterone propionate and estradiol dipropionate given to intact animals inhibited formation of granulation tissue. If growth hormone was injected simultaneously with sex hormone, the former showed stimulating effects in some instances, indicating that the inhibition of formation of granulation tissue was due to suppression of the anterior pituitary by sex hormones. The adrenal cortex exerted its effects in two ways: Desoxycorticosterone acetate (DOCA) injected into intact animals prior and during the turpentine abscess formation had a marked stimulating effect, affecting the fibroblasts and changing the appearance of the ground substance. Cortisone had an inhibitory effect on fibroblasts and inhibited the collagen formation. ACTH had a similar but less pronounced effect.

The antagonism between the effects on connective tissue formation of desoxycorticosterone and of cortisone has also been noted by Selye (1949). The inhibitory effect of cortisone on connective tissue formation takes place in granulation tissue around a turpentine abscess also if cortisone is suspended in turpentine (Shapiro *et al.* 1951). The formation of granulation tissue was completely suppressed in the wounds of mice by cortisone injection (Spain *et al.* 1950). Cortisone also exerts an inhibitory effect on oxygen consumption of granulation tissue (Scarpelli *et al.* 1953). Wound healing is known to be inhibited also by ACTH (Creditor *et al.* 1950). Retardation of all phases of healing of fractures in the rabbit was noted by Blunt *et al.* (1950). Desoxycorticosterone increases the number of fibroblasts in a turpentine abscess (Taubenhaus and Amromin 1950), and increases fibrous reaction to inflammation (Selye 1949). If given to normal animals DOCA leads to increased fibroblastic activity and to increased deposition of metachromatic material (Pirani *et al.* 1951). In tissue culture, DOCA has a deleterious effect on fibroblasts that is potentiated by the addition of cortisone to the culture media (Cornman 1951). The growth hormone of the pituitary has an effect similar to that of DOCA on fibroplasia (Selye 1951, Taubenhaus et Amromin 1950). This effect is opposite to that of ACTH. Growth hormone,

however, has failed to counteract cortisone action in wounds (Spain and Molomut 1953). An investigation of Roberts *et al.* (1951) on chick embryos showed that the only abnormality in the pattern of free amino acids of the tissues, produced by injection of cortisone, was a marked increase in the content of free hydroxyproline. This suggested the authors to conclude that *there may be a specific effect of cortisone on the metabolism of hydroxyproline.*

#### HORMONAL EFFECTS ON MATURE CONNECTIVE TISSUE

It is widely agreed that one of the actions of the adrenal hormones is to regulate protein synthesis. In the dynamic state, there is a continued breakdown of protein into amino acids followed by resynthesis. Ingle (1950) states that  $C_{11}$ -oxysteroids have a catabolic effect on tissue proteins. According to Clark (1953), the antianabolic effect of cortisone is of great significance in the regulation of protein metabolism. Dubrenil and Timiras (1953) state that the effect of cortisone on tissue proteins is catabolic. The investigations of Neuberger *et al.* (1951) with  $^{14}C$ -labelled glycine indicate that tendon collagen in old rats is metabolically almost completely inert. Thus, the stimulatory effect of DOCA on fibroblasts observed by Pirani *et al.* (1951) is considerably less or absent when connective tissue elements have reached partial or almost complete maturity. The presence of changes in granulation tissue, and the lack of them in mature, resting connective tissue, confirms the view that *a profound difference in the response mechanism exists between resting and actively proliferating connective tissue.* Cavallero and Braccini (1951), however, found that in cortisone treated rats the mast cells were clearly reduced in number also in the muscle tissue and heart, and that metachromatically stainable material had practically disappeared, as is the case also in the granulation tissue. The number of mast cells was also decreased after cortisone treatment in the experiments of Asboe-Hansen (1952). Devitt *et al.* (1953), however, did not find any significant changes in the number of mast cells in the rat mesentery, heart, and skeletal muscle after a treatment of animals with cortisone or thyroxine. According to Muller (1951) also the effect of estrogen on the connective tissue of the rat appears to be

selective. Estrogen therapy seemed to bring about an increase in the density and number of collagenous fibres in the dermal layer of the skin, whereas in the loose connective tissue the reverse was the case. In the loose connective tissue, estrogen induced a rejuvenation process, *e.g.* an increase in the immature elements, and in the finely granular appearance of ground substance. That hormones are able to produce changes in young connective tissue was also proved by Aterman (1954), who, using the method of Neuman and Logan, observed that cortisone produced a significant reduction in the amount of collagen (hydroxyproline) of liver in the early stage of fibrosis but had no effect in the later stages. Local cortisone treatment of skin, however, is claimed to cause thinning of the epidermis, and reduction of the number of the epidermal cells (Castor and Baker 1950).

*Collagen and growing.* — Spencer *et al.* (1937) found that the collagen content of gastrocnemius, biceps femoris, and triceps brachii of rabbits is independent of the age of animals. According to Harman (1949) the ventricles of a normal human heart are chemically similar up to the age of 10 years, whereas beyond this age some hearts may contain a significantly increased quantity of collagen. An electron microscopic study of Gross (1950) indicates that the distribution of thickness of collagenous fibrils in the skin of rats from 18 to 60 days of age is irregular, ranging from 400 to 2000 Å, the broader fibrils predominating with advancing age.

On the other hand, numerous studies have been published which deal with the interrelations between the collagen content of different organs and the body weight or the weight of the organ itself. Harkness and Harkness (1954b) found that the collagen content of the liver increases regularly with body weight; liver weight increases less rapidly, liver collagen more rapidly than body weight. Lowry *et al.* (1941), however, did not find evidence for such a steady relationship. Elster and Lowry (1950) observed an increase, followed by decrease, of collagen percentage in the liver of guinea pigs as the body weight increased, simultaneously the collagen percentage in the spleen diminished, and the collagen percentage in the lung increased. Sobel *et al.* (1953) recorded a high correlation of skin collagen content with body weight in rat, higher than that reported by Harkness for the liver.

## EFFECT OF MUSCULAR EXERCISE ON ADRENALS, HEART, AND SKELETAL MUSCLE

*Adrenals.* — Enlargement of adrenals following forced muscular exercise was described in 1904 by Bardier and Bonne, and later among others by Ciovini (1912), Hatai (1915) and Andersen (1935). The enlargement is due to the hypertrophy of adrenal cortex, which can be prevented by hypophysectomy in experimental animals (Hajdu *et al.* 1942, Ingle 1938). The other responses of adrenals to muscular exercise are also characteristic to those of G-A-S: hyperemia during the first hours, that is at a time corresponding to the alarm reaction, and loss of cromaffin granules (Selye 1936); and an initial decrease followed by an increase of ascorbic acid and cholesterol (Ratsimamanga 1939, Sayers and Sayers 1948). There is considerable evidence on that the secretion of corticoids by the adrenals rises above the normal level during muscular work. This was first suggested by Selye and Dosne (1940) who observed considerable quantities of corticoid material after intense muscular exercise in the urine of racing horses. Also in man it has been shown that urinary corticoid excretion is augmented by muscular work (Venning and Browne 1949). Nichols and Miller (1948) found that the sweat induced by muscular exercise contains a significant amount of corticoid material.

*Heart.* — Muscular exercise raises the pulse rate and reinforces contractions of the heart muscle. There is a marked dilatation of arterioles and capillaries in the active muscle while the splanchnic vessels contract (Lee 1949). It is well known that muscular activity is a strong stimulus for the development of cardiac hypertrophy both in athletes (Herxheimer 1929) and in animals, *e.g.* in rats (Hatai 1915). Hypertrophy of the heart is due to the enlargement of existing fibres through an increase of sarcoplasm, without increasing of the number of the fibrils (Maximow and Bloom 1952, Hakkila 1955). There has been disagreement whether the number of capillaries is increased or decreased during hypertrophy of heart due to muscular exercise. According to Petrén *et al.* (1936) the number of heart capillaries increases along with the increase of fiber size. Shipley *et al.* (1937) stated that this is also the case when hypertrophy of the heart is induced by prolonged physical exercise of adult animals, and that no increase of the number of

capillaries accompanies the cardiac hypertrophy brought about by experimental valve lesion. An increase of the number of capillaries during hypertrophy of heart was also found by Sylvén (1938). Several investigators, however, have found a decrease in the number of capillaries in hypertrophied heart when compared with normal hearts (*e.g.* Roberts and Wearn 1941, Frank 1950, Hakkila 1955). Thus, it was suggested that »no multiplication of capillaries takes place in cardiac hypertrophy induced by training, the decreased capillary concentration in cardiac hypertrophy probably being due chiefly to the capillaries pushed farther apart by the hypertrophied muscle fibres» (Hakkila 1955). The papillary muscles and the ventricular septum of the heart of rabbits often show foci of hyalinisation or even necrosis after heavy muscular exercise. This change was ascribed to local anoxemia since it was produced with particular ease by exercise after hemorrhage (Buechner and Lucadou 1934).

*Skeletal muscle.* — The hypertrophy of a repeatedly trained muscle has been known since long. Thus, »the blacksmith's arm reacts with hypertrophy» (Du Bois - Reymond 1882). Also the runner's diaphragm reacts with hypertrophy (Strauss 1930). As in the heart, hypertrophy of a trained skeletal muscle is due to the enlargement of existing fibres through an increase of sarcoplasm (Maximow and Bloom 1952). The increase in the number of capillaries along with the hypertrophy of skeletal muscle has been suggested *e.g.* by Petren *et al.* (1936) and Vannotti and Pfister (1933).

Adrenalectomy notoriously causes intense muscular weakness and ready fatigability. If rats have been previously trained they retain much of their ability to perform intense muscular exercise even if their adrenals are removed. This has been interpreted as showing that it is not the actual presence of adrenal hormones which is needed for the performance of muscular work but that adrenals are mainly needed during the process of adaptation (Selye 1936).

### SUMMARIZING REMARKS

Controversy still exists concerning the manner in which connective tissue is maintained in the body. One view states that there is a dynamic equilibrium in which formation and destruction of connective tissue take place simultaneously and continuously. Another theory maintains that there is a constant anabolism of connective tissue unaccompanied by any appreciable breakdown. Further, it has been claimed that collagenous fibres become metabolically inert with advancing age.

It is widely recognized that the adrenal cortex plays an important role in the regulation of protein metabolism. There is a general agreement that the adrenocortical hormones control the formation of connective tissue. Still, the question remains unanswered whether they also regulate the amount and chemical composition of adult connective tissue in undamaged organism.

Hydroxyproline is an essential amino acid in connective tissue fibres. Relatively little is known about the metabolism of hydroxyproline. Some observations, however, suggest that there may be a specific effect of cortisone on the metabolism of hydroxyproline.

## PROBLEM AND PLAN OF THE PRESENT INVESTIGATION

The present investigation was undertaken in order to throw some light on the relation of hydroxyproline content of muscular organs to the activity of the adrenal glands. The work was performed in 3 separate sections, determining the hydroxyproline content of heart and quadriceps femoral muscle after following treatments of animals:

1) *Stimulation of adrenal cortex* by adrenocorticotrophic hormone. The activity of adrenal glands is increased in G-A-S through increased secretion of this hormone by anterior pituitary. Thus, an effect similar to that produced by treatment with ACTH, ought to be induced by any stimulus which elicits the G-A-S. Muscular exercise was chosen as being of special interest for the present investigation since it is known to cause hypertrophy of skeletal muscles and heart. The increased activity of the organs themselves might have an effect different from that of adrenal hormones on the hydroxyproline content. It is not known what happens to the connective tissue of muscles during muscular hypertrophy.

The animals were trained by letting them swim from 1 to 4 hours daily. After different periods of treatment with adrenocorticotrophic hormone or after repeated muscular exercise the hydroxyproline content of heart and skeletal muscle was determined.

2) *Treatment with different adrenal corticoids*. Animals were injected daily with commercial cortisone, hydrocortisone, or DOCA preparations.

3) *Bilateral adrenalectomy*. The determinations of hydroxyproline were performed 15 days after the operation.

In addition to the determinations of hydroxyproline, some skeletal and heart muscles were analysed for total nitrogen. — The weight of adrenals was recorded as a measure of the adrenal state of the animals.

## MATERIAL AND METHODS

### EXPERIMENTAL ANIMALS

Mice were used in the present study. The animals were 45—90 days of age, and 12—32 gm. of weight. They were of the same strain bred in the colony of the Institute of Occupational Health, Helsinki. The animals were housed in cages, at most 10 mice of same sex and of same age in each, at a constant room temperature. They were fed a standard diet<sup>1)</sup> and allowed to drink tap water *ad libitum*. The mice in each cage were handled as a separate group, experimental or control. Both males and females were included in each control and experimental group, although housed in separate cages.

After the treatment period, the mice were killed by crushing their head. Immediately after the death the carcasses were autopsied, and the adrenal glands were removed and weighed. Heart ventricles were removed, weighed by a torsion balance and restored in a closed weighing bottle. Both quadriceps femoral muscles were removed from the insertions to bone and weighed by torsion balance. About one fourth in both ends of the muscle with the insertion tendons were then discarded. The remaining middle-parts of both muscles without membranous surfaces were weighed and restored into closed weighing bottles, one for the estimation of hydroxyproline content, and the other for determining dry fat-free weight.

<sup>1)</sup> The diet was of the following composition:

Whole wheat meal	600 gm.
Wheat germ	200 gm.
Casein	130 gm.
Margarine	48 gm.
Salt mixture	20 gm.
Cod liver oil	2 gm.

The margarine was melted and mixed with the cod liver oil. This mixture was added in small portions to other constituents of the diet.

## CHEMICAL ANALYSES

## DETERMINATION OF THE HYDROXYPROLINE CONTENT

*Previous methods.* — Until 1950 when Neuman and Logan published their method, no satisfactory method for the determination of hydroxyproline was available. Lang (1933) and Akabori and Waldschmidt-Leitz (1934) had developed a colorimetric estimation involving oxidation to pyrrole with sodium hypochlorite and colour formation with isatin or p-dimethyl-aminobenzaldehyde. However, oxidation was said to be incomplete and a correction factor was necessary. Dakin (1920) and Bergman (1935) estimated hydroxyproline by isolation procedures. These procedures also required the use of large correction factors, and relatively large quantities of protein as well. McFarlane and Guest (1939) devised a colorimetric method for hydroxyproline involving sodium peroxide oxidation and colour formation with copper and isatin. According to Devine (1941), this method yielded low values.

Neuman and Logan claimed that their method is applicable to the determination of hydroxyproline in hydrolysates of 40 to 100  $\gamma$  of collagen with a reproducibility of 2 per cent and an accuracy of  $\pm 2$  per cent as judged by recovery of hydroxyproline from elastin hydrolysates and from an amino acid mixture simulating collagen. Oxidation in the manner of McFarlane and Guest with sodium peroxide yields products that form an intense red colour with p-dimethylaminobenzaldehyde. The intensity of colour produced with 5 to 15  $\gamma$  of hydroxyproline is 4 to 5 times that formed in previous colorimetric procedures. Because of greater intensity of colour and different preliminary treatment of the hydroxyproline solutions, the amount of protein required is only 1 to 2 per cent of that in earlier methods.

In acid hydrolysates of proteins, the only amino acid except hydroxyproline which gives colour under the conditions employed is tyrosine, which yields 1.5 per cent as much colour as hydroxyproline. This interference is so small that it can be corrected from the tyrosine content of the protein. Pure tryptophan gives 0.7 per cent as much colour as hydroxyproline but humin formation on acid hydrolysis of proteins eliminates this interference. Tissues low in collagen (muscle, kidney, spleen,) however, require a pre-

liminary extraction of non-collagenous proteins to avoid great amounts of the extraneous tyrosine-containing proteins. In Neuman and Logan's experiments (1950) tyrosine-containing proteins were present in extracts made by autoclaving whole tissues of collagen content (muscle, liver, spleen, brain, kidney) in amounts sufficient to produce colour equivalent to 2 to 9 per cent of that from the hydroxyproline. After preliminary extraction of the non-collagenous proteins with 20 per cent urea solution, the tyrosine remaining usually accounted for much less than 2 per cent of the total colour formation, except in the case of muscle tissue, especially heart muscle, in which the remaining tyrosine colour amounted to almost 3 per cent of the total.

According to Neuman and Logan their method gives no significant amount of colour with proline. They found that three commercial preparations of L-proline yielded colour corresponding to 2,3 to 3,4 per cent of hydroxyproline. When proline had been purified previously the colour formation with p-dimethylaminobenzaldehyde decreased to 0,17 per cent of that produced by hydroxyproline. Neuman and Logan claimed that the colour formation of commercial proline preparations was due to the presence of hydroxyproline.

Neuman and Logan noted that the hydroxyproline content of most gelatins and collagens was rather constant, 13 to 14 per cent. (13 to 14 mg. of hydroxyproline per 100 mg. of dry fat-free protein). The range of these values in the literature is 12 to 14.6 per cent of gelatin (Lang 1933, Dakin 1926, Bergman 1935, McFarlane and Guest 1933, Fürth and Minnibeck 1932) as estimated with other methods.

The constancy of the hydroxyproline content of collagen has lead to an extensive use of Neuman and Logan's method for determination of collagen content of different organs or tissue. (Harkness and Harkness 1954, 1955, Consden *et al.* 1952, Gustavson 1955, Neuman and Logan 1950, Aterman 1954 *etc.*). Thus, Neuman and Logan's method has displaced the older methods, *e.g.* the gravimetric procedure of Lowry, Killigan and Katersky (1941) and the method of Abercrombie and Johnsson (1946) in wick the nitrogen content of into gelatin converted collagen was determined after the extraction of non-collagenous proteins, the method of Spencer *et al.* (1937) which also was based

on conversion of collagen to gelatin followed by the determination of nitrogen of the gelatin, and the procedure described by Mitchell *et al.* (1926) in which the collagen nitrogen was determined after hydrolysis of tissue by autoclaving and tryptic digestion.

*The method in the present study.*— A few modifications were made to the original method of Neuman and Logan, in order to improve the accuracy of estimations. While the tissue samples were of small quantity, attempts were to be made to avoid loss of substances during the procedures. Thus, the tissue samples were thoroughly minced in an all-glass homogenizer of the Potter-Elvehjem type (1936) instead mincing them with sand in a mortar, as in the original method. The substance containing hydroxyproline consists of thin membranes and tendons, which easily stick to the glassware. Therefore, extraction of non-collagenous proteins, hydrolysis of the residue, and neutralization of the acid hydrolysate were performed in the same centrifuge tube without transferring the mixture from one tube into another as in the original method.

The tissue sample of 30 to 170 mg. of wet weight was thoroughly homogenized in 2.0 ml. of 20 per cent urea solution, and the mixture was transferred into a centrifuge tube. The homogenizer was washed twice with 2.0 ml. of 20 per cent urea solution and the washings were transferred to the centrifuge tube. The tube was allowed to stand for 1 hour, with occasional stirring, and the mixture was centrifuged until clear. The remainings were extracted with another 6.0 ml. of 20 per cent urea solution for one hour, and centrifuged. The remainings were washed 3 times with 6.0 ml. of distilled water at 20° C. The tube with its contents was dried at 60° C, and 1.0 ml. of 6 N hydrochloric acid was added<sup>1)</sup>. The tube was covered with a glass-cap and placed into an autoclave with 47 other similar tubes containing tissue homogenates from both experimental and control animals. The tubes were autoclaved for 3 hours at 3.5 kg./sq.cm. pressure.

The hydrolysates were then neutralized by adding 2.0 ml. of 2.5 N sodium hydroxide solution and thereafter dropwise 0.5 N sodium hydroxide solution using small pieces of litmus paper as indicator. The neutralized solution was filtered into a graduated

<sup>1)</sup> The autoclave was constructed from a discarded gas cylinder by Oy AGA Ab., Helsinki on the basis of a design by Dr. M. J. Karvonen, Mr. J. Puukko, and the author.

glass-cylinder. The filter paper was washed with distilled water until the glass-cylinder contained 10.0 ml. of solution. The solution was shaken and transferred into a test-tube.

After neutralizing all the hydrolysates and transferring them into test-tubes, the photometric estimation of hydroxyproline took place. With accurate pipettes the reagents were distributed as follows:

*Tube 1.* 1.0 ml. of distilled water;

*Tubes 2 and 3.* 1.0 ml. of standard containing 5  $\gamma$  of hydroxyproline;

*Tubes 4 and 5.* 1.0 ml. of standard containing 10  $\gamma$  of hydroxyproline;

*Tubes 6 and 7.* 1.0 ml. of standard containing 15  $\gamma$  of hydroxyproline;

A sample of 1.0 ml. of each hydrolysate was pipetted into two test tubes for duplicate estimations of hydroxyproline. In succession 1.0 ml. each of 0.01 M copper sulphate solution, 2.5 N sodium hydroxide, and 6 per cent hydrogen peroxide were pipetted into each test tube.

The solutions were stirred and shaken occasionally during a period of 5 minutes and were then held in a water bath at 80° C for 5 minutes, shaking them frequently. According to Neuman and Logan, heating and shaking destroy the excess of peroxide. Traces of peroxide would decrease colour formation and produce an orange red hue. Then, the tubes were chilled in an ice and water bath. 4.0 ml. of 3.0 N sulfuric acid was added dropwise; 2 ml. of 5 per cent p-dimethylaminobezaldehyde solution, in n-propanol prepared according to Neuman and Logan, was then added with thorough mixing.

The tubes were placed in a water bath at 70° C for 16 minutes and then cooled in tap water. The contents were transferred to selected cuvettes, and light extinction was read with a Coleman Spectrophotometer at 540  $\mu\mu$ . Tube no 1 was used for the blank setting. The amount of hydroxyproline (measured in micrograms in 1.0 ml. of unknown solution) was established by finding the point corresponding to its optical density on the standard curve prepared at the same time. The percentage concentration of

hydroxyproline in the sample of material was determined by the equation

$$\frac{\text{Micrograms of hydroxyproline in 1.0 ml. of unknown solution}}{\text{Micrograms of sample in 1.0 ml. of unknown solution}} \times 100$$

As mentioned, the other quadriceps femoral muscle sample of each animal was stored up for the determination of dry fat-free weight. The weighed sample was placed into a test tube filled with acetone. After six hours or more the acetone was decanted and replaced by 15.0 ml. of fresh acetone which was allowed to stand for another 6 hours or more. This was followed by extraction with 15.0 ml. of ether for 12 to 16 hours. The residue was dried to a constant weight at 108° C. The relation dry fat-free weight/fresh weight was expressed as a percentage.

#### CONTROL OF THE METHOD USED FOR DETERMINATION OF HYDROXYPROLINE

1. In order to find out the degree of the hydrolytic release of hydroxyproline from protein, 20 tubes containing tissue homogenate, after autoclaving for 3 hours at 3,5 kg./sq.cm. pressure, were washed with distilled water and autoclaved once more with 1.0 ml. of 6 N hydrochloric acid.

No colour formation took place when the autoclaved washing solutions were neutralized and treated with p-dimethylaminobenzaldehyde. This indicates that the first autoclaving of homogenates for 3 hours at 3,5 kg./sq.cm. pressure with 1.0 ml. of 6 N hydrochloric acid *released hydroxyproline completely.*

2. The water used in autoclave for producing steam was examined for hydroxyproline. After autoclaving tissue homogenates, the water proved to contain no hydroxyproline. Thus, *all hydroxyproline had remained in the tubes.*

3. Colorimetric comparison was made between 1.0 ml. each of standard solution and 1.0 ml. of the same solution after autoclaving it with 1.0 ml. of 6 N hydrochloric acid.

The amount of hydroxyproline of autoclaved standards did not differ from those of non-autoclaved standards. This indicates that *no change occurred in hydroxyproline during autoclaving.*

The standard error of a single determination, often called »the method error«, was 2.7 per cent, as estimated on the basis of 40 pairs of determinations. While deviations larger than twice the standard error are likely to occur in at most 1 case in 20, the accuracy of the method can suitably be described as  $\pm 5.4$  per cent.

#### CORRECTION OF HYDROXYPROLINE VALUES FOR TYROSINE

As previously mentioned, the only amino acid which produces colour with p-dimethylaminobenzaldehyde under the conditions employed, is tyrosine which yields 1.5 per cent as much colour as hydroxyproline. The tyrosine content of hydrolysed extracts was determined with the aid of Folin and Ciocalteu's phenol reagent according to the method described by Herriot (1941).

One ml. of hydrolysed and neutralized extract was pipetted into a test tube along with 1.0 ml. of M/100 copper sulfate and 8.0 ml. of 0.5 N sodium hydroxide. To this was then added dropwise 3.0 ml. of the 1/3 dilution of Folin's and Ciocalteu's reagent, just diluted, whirling simultaneously. The colour was then read with Coleman Spectrophotometer at 650  $\mu\mu$  after 5 to 10 minutes against a blank setting with distilled water instead of hydrolysed extract treated in a similar way. For the standard curve, solutions containing 0.01 mg., 0.1 mg., and 1.0 mg of tyrosine in 1.0 ml. of water were treated in a similar way.

Correction of hydroxyproline content of each sample was made according to the equation:

$$Hy_c = Hy - \frac{1.5 \cdot Ty}{100}$$

$Hy_c$  = corrected hydroxyproline content ( $\gamma$ /mg.)

$Hy$  = uncorrected hydroxyproline content ( $\gamma$ /mg.)

$Ty$  = tyrosine content ( $\gamma$ /mg.)

This corrected value of hydroxyproline content of heart is given as per cent of fresh weight of heart muscle. From the skeletal muscles, the values are given as per cent of dry fat-free weight.

The average correction for tyrosine was 3.0 per cent in heart, and 1.3 per cent in quadriceps femoral muscle.

## STATISTICAL ANALYSIS

In both control and experimental groups a few statistical characteristics were calculated as follows. To describe the general tendency of observations, the mean

$$(1) \quad \bar{x} = \sum x / n$$

was computed. In the formula  $x$  stands for numerical observations,  $n$  being the number of them. Variation of the material is described by the standard deviation

$$(2) \quad s = \sqrt{(\sum x^2 - (\sum x)^2/n) / (n-1)},$$

and the standard error of the mean

$$(3) \quad s_{\bar{x}} = s/\sqrt{n}$$

measures statistical precision of the mean.

In comparing two means  $\bar{x}_1$  and  $\bar{x}_2$  the difference of them was calculated:

$$(4) \quad \Delta = \bar{x}_1 - \bar{x}_2$$

In part I of the present study a sufficiently accurate estimate of the standard error of the difference of two means was obtained by

$$(5) \quad s_{\Delta} = \sqrt{s_{\bar{x}_1}^2 + s_{\bar{x}_2}^2}$$

In parts II and III of the present investigation a more accurate method was applied. A pooled estimate of the standard deviation was formed taking simultaneously into consideration variability in the entire material, variability between different groups, however, excluded. Formula (2) was in use, with the modification that the numerator was the sum of numerators computed separately within each group, and that, similarly, the denominator was the sum of denominators computed within each group

separately. On the basis of this over-all estimate of standard deviation,  $s$ , the standard error of the difference of any two means was then calculated as follows:

$$(6) \quad s_{\Delta} = s \sqrt{(n_1 + n_2) / n_1 n_2}$$

Statistical significance of the difference between two means was then tested by calculating the test variable  $t$ :

$$(7) \quad t = (\bar{w}_1 - \bar{x}_2) / s_{\Delta}.$$

If this quantity is larger than can be expected on the basis of chance only, the difference is said to be significant.

Whether this is the case or not, was determined by consulting appropriate statistical tables (Fisher and Yates 1953), which reveal the probability  $P$  of that the difference would have been arisen by chance only. If this probability is small, say 0.05 at most, the difference usually will be taken as significant, though even in such cases the conclusion may sometimes be wrong. In reporting results of the  $t$ -test, the  $t$ -value itself together with  $P$  are given. If  $P$  exceeds 0.05, its numerical value has been omitted and replaced by two dots (. .). The procedure described above applied when comparing means of experimental groups with those of control groups. However, as the hydroxyproline content of an organ may be dependent on the weight of the organ, an alternative testing procedure was applied in order to eliminate possible effects of this dependence. Therefore, regression equations which reveal the numerical dependence mentioned were calculated on the basis of the control series. When applying regression analysis, it was assumed that the hydroxyproline content  $y$  can, on an average, be estimated as a linear function of the weight of then organ,  $x$ , thus:

$$(8) \quad y \simeq Y = a + bx.$$

In formula (8) the symbols  $a$  and  $b$  stand for constants which were determined by applying the method of least squares on the data. Numerical values of these constants are given on pages 32 and 46.

Then, for each experimental group separately, the regression estimate  $Y$  of hydroxyproline content was computed, and the

actual average content was compared with this estimate. For such a comparison, the standard error of the regression estimate  $Y$  was calculated with the aid of the formula

$$(9) \quad s_Y = s_C \sqrt{(1/n_C - (\bar{w}_E - \bar{w}_C)^2 / Q_{wC})},$$

where  $s_C$  = so-called residual standard deviation of hydroxyproline content in the control group after the regression line is fitted,

$n_C$  = number of animals in the control group,

$\bar{w}_E, \bar{w}_C$  = average weight of organ in experimental and control group, respectively,

$Q_{wC}$  = so-called sum of squares for weight of the organ in the control group.

Then, the  $t$ -test was performed in analogy with the presentation in connection with formulas (4) and (5), noting, however, that in this instance there are  $n_C + n_E - 3$  degrees of freedom available.

Regression analysis is illustrated in Figs. 1, 2, 7 and 9 which show the regression lines represented by formula (8) together with actual observed values in control material, and the averages of experimental groups in relation to this line.

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## RESULTS



## I. STIMULATION OF ADRENAL CORTEX

In this part of work the function of adrenal cortex was stimulated, directly, with the aid of adrenocorticotrophic hormone and, further, indirectly, by muscular exercise. The stimulating effect of the latter on adrenal cortex is known to be mediated through an increased production of adrenocorticotrophic hormone by the pituitary gland.

### A. MUSCULAR EXERCISE

#### EXPERIMENTAL

It is rather difficult to put an animal into work. In preliminary experiments some forms of muscular exercise were tested. Running on a treadmill seemed to be constant and rather heavy work. The animals, however, were able to run not longer than for a quarter of an hour before they began to stumble and were hurt. Swimming seemed to be easier for mice. The disadvantage of this form of exercise was the tendency of animals to float with minimal movements of the extremities. Some of the animals were able to swim up to 4 hours before they were exhausted and drowned.

During the exercise period, the animals were once a day put into a tub filled with 38° C water, which temperature was maintained with a thermostat. On the first day of exercise, the animals were let to swim 1/4 to 1/2 hours depending on their condition. When first time put in water some of the animals got so excited that they exerted all their strength in fifteen minutes. In 2 or 3 days they generally learned to swim for one hour or more.

In all 117 mice were included in the exercise experiments, 31 of which were used for control. The following table summarizes the treatment of animals in different groups and shows the numbering of the groups to be used in subsequent presentation:

- I. 31 mice as a control group
- II. 24 mice swam 1 hour daily during 5 days
- III. 26 mice swam 1 hour daily during 10 days
- IV. 9 mice swam 1 hour daily during 5 days and 2 hours daily during the subsequent 5 days
- V. 19 mice exhausted daily by swimming up to 4 hours during 10 days
- VI. 8 mice exercised as the group IV., but killed one week after the exercise period.

The animals were killed and dissected the day after the last exercise. Chemical analyses of both experimental and control group were made simultaneously in order to avoid differences in experimental conditions.

## RESULTS

*Weight of Animals.* — On the average, the animals trained by swimming lost weight 1 to 5 grammes (Table 1). Most marked weight loss was observed in group IV, next to which came group III. Although small weight loss was observed in groups V and VI, the loss was not statistically significant as it was in groups II, III and IV.

TABLE 1. *Weight of the animals, gm.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
I	31	20.3	2.45	0.44	.	.
II	24	17.8	3.36	0.68	3.18	0.01
III	26	16.9	2.86	0.56	4.41	0.001
IV	9	14.9	1.42	0.47	4.89	0.001
V	19	19.4	3.60	0.80	1.04	..
VI	8	18.3	1.31	0.46	1.72	..

*Weight of Heart.* — Peculiarly in most of the trained groups an average decrease of 2 to 15 mg. in heart weight was observed, though only the most marked decrease in group IV was statistically significant (Table 2). However, the average heart weight increased by more than 21 mg. in group V, the change being highly significant.

TABLE 2. *Weight of heart, mg.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
I	25	94.0	17.8	3.6	.	.
II	23	92.1	15.6	3.3	0.42	..
III	22	93.5	16.2	3.5	0.11	..
IV	9	78.7	7.0	2.3	2.51	0.05
V	17	115.5	16.7	4.0	4.39	0.001
VI	8	82.2	7.2	2.5	1.87	..

*Weight of Quadriceps Femoral Muscle.* — Observations concerning the weight of quadriceps femoral muscle were largely similar to those concerning the weight of heart (Table 3). Also here group V represents a marked deviation from the general tendency of observations.

TABLE 3. *Weight of quadriceps femoral muscle, mg.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
I	31	49.5	10.1	1.8	.	.
II	24	46.9	11.5	2.4	0.93	..
III	26	47.9	9.6	1.9	0.59	..
IV	9	39.0	3.0	1.0	2.69	0.05
V	19	66.2	12.6	2.9	5.57	0.01
VI	8	45.1	6.0	2.1	1.10	..

*Relative Weight of Heart.* — Training obviously caused an increase in the relative weight of heart (Table 4), the changes varying from 10 to 30 per cent. The rest period allowed for group VI seemed to cause the relative weight of heart to return to its normal level.

*Relative weight of quadriceps femoral muscle.* — In the relative weight of quadriceps femoral muscle the effect of training was similar to that in the relative weight of heart (Table 5), though the increase in group II failed to obtain statistical significance.

TABLE 4. *Relative weight of heart, mg./100 gm.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparisson with the control group	
					<i>t</i>	<i>P</i>
I	25	465	48	10	.	.
II	23	538	125	26	2.63	0.02
III	22	557	81	17	4.68	0.001
IV	9	522	51	17	2.94	0.01
V	17	602	70	17	6.99	0.001
VI	8	449	23	8	1.27	..

TABLE 5. *Relative weight of quadriceps femoral muscle, mg./100 gm.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
I	31	243	27	4.8	.	.
II	24	269	71	14	1.67	..
III	26	296	64	13	3.98	0.001
IV	9	262	16	5.4	2.60	0.02
V	19	343	36	8.2	10.5	0.001
VI	8	246	18	6.2	0.32	..

*Correlation of hydroxyproline concentration and weight of organ.* — In order to reveal the possible relation between the weight of the organ, and its hydroxyproline concentration, a regression analysis was performed using as material the control group I. In such an analysis, the constants *a* and *b* in the regression equation

$$Y = a + b x$$

are determined by applying the method of least squares. In the equation above, *Y* stands for the «calculated» or regression value of concentration, *x* for the weight of the organ. The computations resulted in the following two equations:

1) *Hydroxyproline concentration in heart* ( $\gamma/\text{mg}$ ) =  $0.61 - 0.0032 \times \text{weight of heart (mg)}$ ;

2) *Hydroxyproline concentration in quadriceps femoral muscle* ( $\gamma/\text{mg. dry fat-free weight}$ ) =  $6.39 - 0.0064 \times \text{weight of quadriceps femoral muscle (mg.)}$ .

Both regressions are highly significant ( $P = 0.001$ ), and the lines represented by the equations are shown in Figs. 1 and 2. together with actual individual values in the control group.

Besides of comparing the average concentration of any group with that of the control group, a regression line value was calculated for each trained group by replacing for the weight of organ in the above equations the average weight of the organ in the group, whereafter the average concentration of the group was compared with that calculated on the basis of regression. This latter comparison enabled one to eliminate the possible effect on concentration of the weight of the organ.

*Hydroxyproline concentration of heart.* — Irregular results were obtained concerning the hydroxyproline concentration in heart (Table 6). In groups IV and VI the concentration increased, in the remaining groups a decrease took place. Only the increase in group IV was statistically significant as compared with control level. In comparison to the regression line defined in the foregoing, none of the groups showed significant deviations (Fig. 3).

TABLE 6. *Hydroxyproline concentration of heart,  $\gamma$ /mg.*

Group	Number of animals	Mean conc.	Stand. dev. of conc.	Stand. error of the mean	Comparison with the control group		Comparison with the regression line	
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
I	25	0.30	0.084	0.017	.	.	.	.
II	23	0.29	0.115	0.024	0.69	..	1.10	..
III	22	0.30	0.096	0.020	0.04	..	0.41	..
IV	9	0.41	0.094	0.031	2.94	0.01	1.41	..
V	17	0.25	0.058	0.014	1.82	..	0.41	..
VI	8	0.34	0.065	0.023	0.95	..	0.36	..

*Hydroxyproline concentration of quadriceps femoral muscle.* — In quadriceps femoral muscle the hydroxyproline concentration generally increased because of training, save in group II where a small decrease took place. However, as compared with the control level, the increase in group IV only was statistically significant. On the other hand, not group IV but groups II and V showed a statistically significant deviation from the regression line of concentration (Fig. 4).

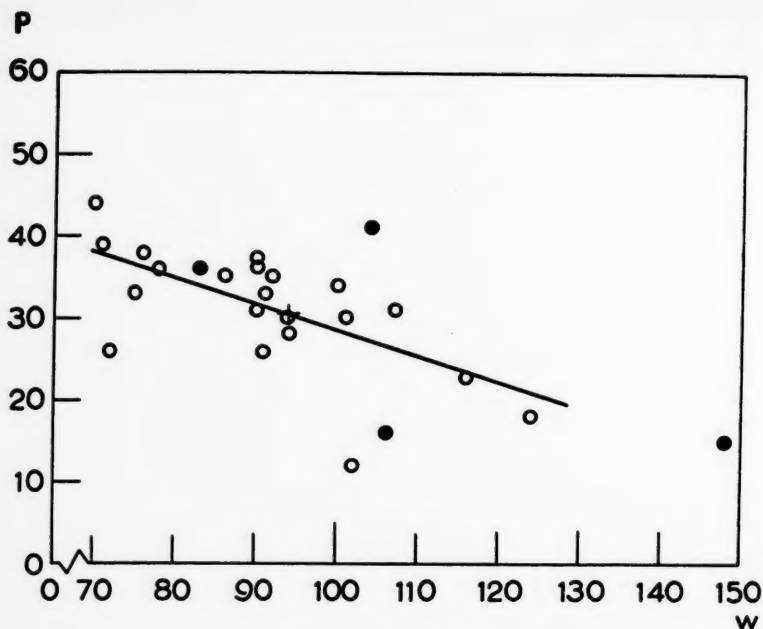


FIG. 1. Hydroxyproline concentration (P) as  $\gamma$ /mg.  $\times 100$  and absolute weight (w) as mg. of heart in untreated control animals. Circles indicate the individual values of females and dots those of male animals. The regression line corresponds to the equation: hydroxyproline concentration =  $0.61 - 0.0032 \times \text{weight of heart}$ .

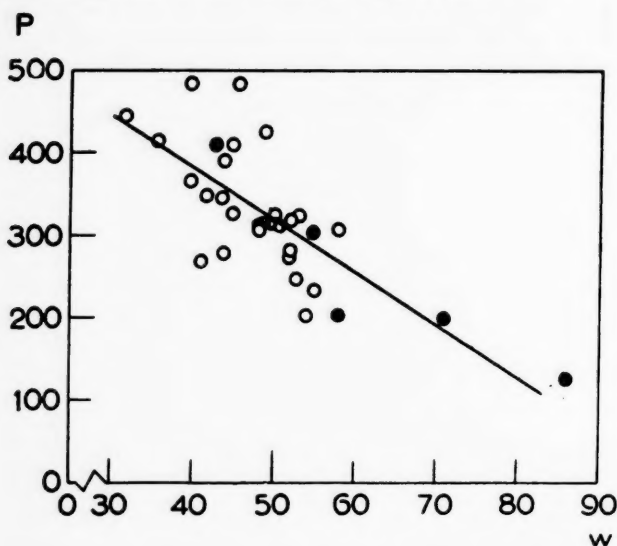


FIG. 2. Hydroxyproline concentration (P) as  $\gamma$ /mg.  $\times 100$ , and absolute weight (w) as mg. of quadriceps femoral muscle in untreated control animals. Circles indicate the individual values of female animals, and dots those of male animals. The regression line corresponds to the equation hydroxyproline concentration =  $6.39 - 0.0064 \times \text{weight of quadriceps femoral muscle}$ .

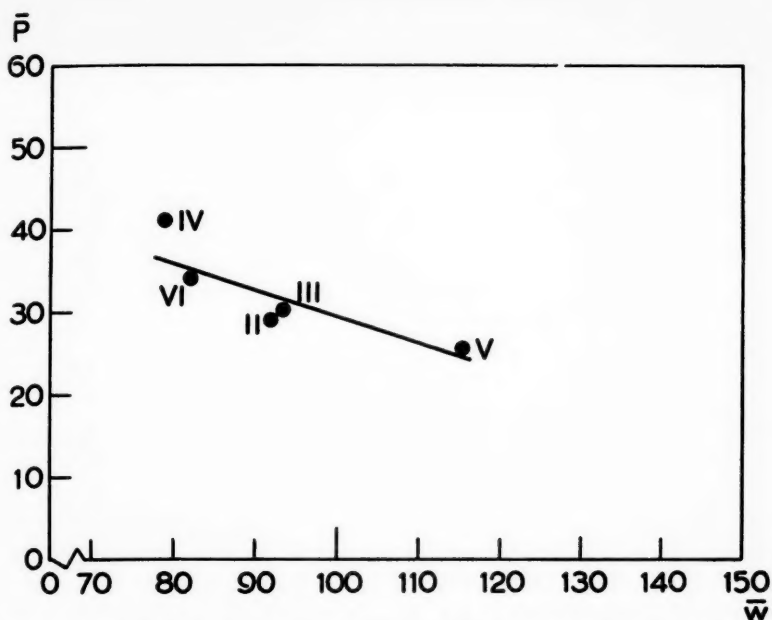


FIG. 3. Deviations of the mean hydroxyproline concentrations and absolute weights of heart in trained groups from the regression line of the untrained control group. The numbering of groups is the same as in the text. None of the deviations proved to be statistically significant.

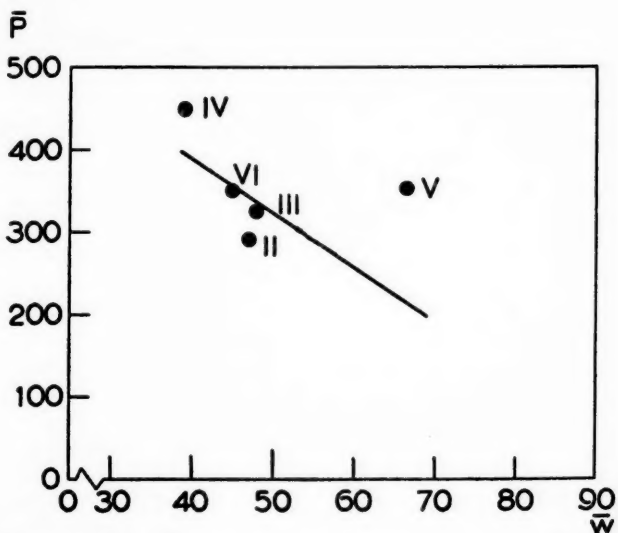


FIG. 4. Deviations of the mean hydroxyproline concentrations ( $\bar{P}$ ) and absolute weights ( $\bar{w}$ ) of quadriceps femoral muscle in trained groups from the regression line of the untrained control group. The numbering of groups same as in the text. The deviations of groups II and V were statistically significant.

TABLE 7. *Hydroxyproline concentration in quadriceps femoral muscle,  $\gamma$ /mg.*

Group	Number of animals	Mean conc.	Stand. dev. of conc.	Stand. error of the mean	Comparison with the control group		Comparison with the regression line	
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
I	31	3.22	0.845	0.152	.	.	.	.
II	24	2.92	0.690	0.141	1.03	..	2.70	0.01
III	26	3.26	1.045	0.205	0.11	..	0.26	..
IV	9	4.48	0.865	0.288	3.00	0.01	1.83	..
V	19	3.63	1.963	0.450	1.25	..	3.01	0.01
VI	8	3.63	0.449	0.159	0.92	..	0.67	..

## B. TREATMENT WITH ADRENOCORTICOTROPHIC HORMONE

### EXPERIMENTAL

17 mice were treated with daily injections of 1 int. unit of ACTH, and 15 animals were used as control. The commercial preparation CORTROPHINE «ORGANON» was used. 5.0 ml. of solvent included within the original package was added into a vial containing 25 i.u. of Cortrophine. The solution was stored at a temperature of about  $+5^{\circ}\text{C}$ . Daily subcutaneous injections of 0.2 ml. of thoroughly stirred ACTH-containing solution were made with sterile needles and syringe in the back of the mice. The control animals were treated with daily injections of 0.2 ml. of the pure solvent.

10 of the animals treated with ACTH and 10 control animals were killed after 5 days and the remaining animals after another 5 days' period of treatment.

### RESULTS

*Weight of animals.* — No significant change in the body weight of animals was observed during the treatment with ACTH.

TABLE 8. *Weight of the animals, gm.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control ACTH 5 × 1 i.u.	10	21.5	1.8	0.58	.	.
	10	22.4	4.1	1.3	0.63	..
Control ACTH 10 × 1 i.u.	5	22.1	2.4	1.1	.	.
	7	21.9	3.2	1.2	0.12	..

*Relative weight of heart, and quadriceps femoral muscle.* — No changes took place in the relative weight of heart or quadriceps femoral muscle. (Tables 9 and 10).

TABLE 9. *Relative weight of heart, mg/100 gm.*

Group	Number of animals	Mean weight	Standard deviation of relative weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	8	526	48	17	.	.
ACTH 5 × 1 i.u.	7	502	49	19	1.00	..
Control	4	543	64	32	.	.
ACTH 10 × 1 i.u.	5	468	60	27	1.80	..

TABLE 10. *Relative weight of quadriceps femoral muscle, mg/100 gm.*

Group	Number of animals	Mean weight	Standard deviation of relative weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	10	267	45	14		.
ACTH 5 × 1 i.u.	10	295	41	13	1.45	..
Control ....	5	254	20	9	.	.
ACTH 10 × 1 i.u.	7	271	41	15	0.96	..

*Hydroxyproline concentration of heart.* — An increase of mean hydroxyproline concentration was observed after treatment with ACTH. This change, however, was significant in the group treated during 5 days only. (Table 11.)

TABLE 11. *Hydroxyproline concentration of heart,  $\gamma$ /mg.*

Group	Number of animals	Mean conc.	Standard deviation of conc.	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	7	0.22	0.057	0.022	.	.
ACTH $5 \times 1$ i.u.	7	0.32	0.032	0.012	3.93	0.01
Control	4	0.18	0.036	0.018	.	.
ACTH $10 \times 1$ i.u.	5	0.21	0.058	0.026	0.95	..

*Hydroxyproline concentration of quadriceps femoral muscle.* — The mean hydroxyproline concentration was significantly increased after treatment with 1 i.u. of ACTH during 10 days. In 5 days' period of treatment no significant change could be observed. (Table 12.)

TABLE 12. *Hydroxyproline concentration of quadriceps femoral muscle,  $\gamma$ /mg.*

Group	Number of animals	Mean conc.	Standard deviation of conc.	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	10	3.11	0.57	0.18	.	.
ACTH 1 i.u. $5 \times 1$ i.u.	8	3.41	0.54	0.19	1.14	..
Control	5	2.59	0.46	0.20	.	.
ACTH 1 i.u. $5 \times 1$ i.u.	6	3.23	0.13	0.52	3.03	0.02

## II. TREATMENT WITH ADRENOCORTICAL HORMONES

### EXPERIMENTAL

In this section altogether 104 mice were used, of which 64 as experimental, and 40 as control animals. The experimental animals were treated with daily intramuscular injections of adrenocortical hormones, as the control animals were injected with physiological saline. The injections were made aseptically in dorsal muscle after shaving and cleansing the skin with diluted alcohol.

From the group of *glucocorticoids*, 11-dehydro-17-hydroxycorticosterone-21-acetate, cortisone, CORTONE» MERCK», was used. In the initial experiments, animals were injected with 2.0 mg. of hormone daily during a period of 10 days. While cortisone seemed to cause changes in the hydroxyproline content of muscle, further groups of experimental animals were treated with larger doses of cortisone, and the effect of a treatment with 2.0 mg. during 5 and 15 days also was studied. — Because hydrocortisone is physiologically closely connected with cortisone, a group of animals was treated daily with 2.0 mg. of hydrocortisone, HYDROCORTONE »MERCK», during 10 days. From the group of *mineralocorticoids*, desoxycorticosterone acetate, DOCA »ORGANON», was used in a daily dose of 2.0 mg. during 10 days.

Thus, the following experimental groups were used:

- |    |    |      |         |        |    |      |      |     |     |    |                |                      |
|----|----|------|---------|--------|----|------|------|-----|-----|----|----------------|----------------------|
| A. | 10 | mice | treated | during | 5  | days | with | 2.0 | mg. | of | cortisone      | (10 mice as control) |
| B. | 11 | »    | »       | »      | 10 | »    | »    | 2.0 | mg. | of | cortisone      | ( 4 mice as control) |
| C. | 10 | »    | »       | »      | 15 | »    | »    | 2.0 | mg. | of | cortisone      | (10 mice as control) |
| D. | 8  | »    | »       | »      | 5  | »    | »    | 4.0 | mg. | of | cortisone      | (10 mice as control) |
| E. | 9  | »    | »       | »      | 5  | »    | »    | 8.0 | mg. | of | cortisone      | (10 mice as control) |
| F. | 10 | »    | »       | »      | 10 | »    | »    | 2.0 | mg. | of | hydrocortisone | (10 mice as control) |
| G. | 6  | »    | »       | »      | 10 | »    | »    | 2.0 | mg. | of | DOCA           | ( 6 mice as control) |

The groups A. and F. had common controls like also the groups D. and E. The controls received injections of physiological saline, except those of the group G. which were injected daily with 0.3 ml. of arachidone oil.

#### RESULTS

The results of each experimental group are compared to those of a control group of same family. The chemical analyses of both groups were performed simultaneously.

*Weight of animals.* — A significant decrease of weight was observed in animals treated during 5 days with 4 mg. or 8 mg. of cortisone daily. In the group which was treated with 2 mg. of DOCA during 10 days, a significant increase of weight took place. (Table 13.)

TABLE 13. *Weight of the animals, gm.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	10	24.6	4.3	1.4	.	.
Cortisone 5 × 2 mg.	10	21.7	4.4	1.4	1.50	..
Hydrocortisone 10 × 2 mg.	10	21.2	3.6	1.1	1.93	..
Control	4	20.2	3.4	1.7	.	.
Cortisone 10 × 2 mg.	11	18.7	3.1	0.93	0.77	..
Control	10	23.7	4.6	1.5	.	.
Cortisone 15 × 2 mg.	10	20.9	4.2	1.3	1.42	..
Control	10	21.5	1.8	0.58	.	.
Cortisone 5 × 4 mg.	8	18.2	2.7	0.96	2.92	0.01
Cortisone 5 × 8 mg.	9	17.4	2.1	0.69	4.54	0.001
Control	6	19.5	0.84	0.34	.	.
DOCA 10 × 2 mg.	6	23.5	1.6	0.67	5.31	0.001

*Relative weight of heart.* — The relative weight of heart was increased in all cortisone and hydrocortisone treated groups. (Table 14). This increase seemed to be somewhat proportional to the time of treatment with a constant dose of cortisone.

TABLE 14. *Relative weight of heart, mg/100 gm.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	8	494	42	15	.	.
Cortisone 5 × 2 mg.	8	582	78	28	2.83	0.02
Hydrocortisone 10 × 2 mg.	8	628	47	17	6.03	0.001
Control	3	501	105	61	.	.
Cortisone 10 × 2 mg.	10	654	92	29	2.28	0.05
Control	8	464	24	8.6	.	.
Cortisone 15 × 2 mg.	8	626	99	35	4.48	0.001
Control	8	526	48	17	.	.
Cortisone 5 × 4 mg.	6	709	60	24	6.17	0.001
Cortisone 5 × 8 mg.	7	635	132	50	2.06	0.10
Control	5	571	29	13	.	.
DOCA 10 × 2 mg.	5	535	27	12	2.06	..

*Relative weight of quadriceps femoral muscle.* — The average relative weight of quadriceps femoral muscle showed no significant changes, except in the groups which had been treated with largest daily doses of cortisone. In these groups, contrary to weight of the heart, a significant decrease of the relative weight of quadriceps femoral muscles took place. (Table 15.)

TABLE 15. *Relative weight of quadriceps femoral muscle, mg/100 gm.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	10	254	43	14	.	.
Cortisone 5 × 2 mg.	10	242	33	10	0.74	..
Hydrocortisone 10 × 2 mg.	10	245	44	14	0.46	..
Control	4	279	51	25	.	.
10 × 2 mg.	10	232	61	19	1.47	..
Control	10	271	30	10	.	.
Cortisone 15 × 2 mg.	10	263	41	13	0.51	..
Control	10	267	45	14	.	.
Cortisone 5 × 4 mg.	8	212	29	10	3.15	0.01
Cortisone 5 × 8 mg.	9	222	37	12	2.42	0.05
Control	6	333	26	11	.	.
DOCA 10 × 2 mg.	6	316	25	10	1.21	..

*Hydroxyproline concentration of heart.* — As in the first part of experiments, irregular results were obtained concerning the hydroxyproline content of heart. The only significant changes took place in animals which had been treated with cortisone or hydrocortisone during 5 days. The daily dose of 2 mg. of cortisone or hydrocortisone caused a decrease, whereas the daily dose of 8 mg. of cortisone caused a nearly significant increase when compared with age controls. No significant change, however, took place when the hydroxyproline concentration was compared with the regression line. (Table 16 and Fig. 8.)

TABLE 16. *Hydroxyproline concentration of heart,  $\gamma$ /mg.*

Group	Number of animals	Mean conc.	Standard deviation of concentration	Standard error of the mean	Comparison with the control group		Comparison with the regression line	
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
Control	8	0.23	0.065	0.023	.	.	.	.
Cortisone 5 $\times$ 2 mg.	8	0.17	0.047	0.017	2.20	0.05	0.78	..
Hydrocortisone 10 $\times$ 2 mg.	8	0.17	0.036	0.013	2.35	0.05	0.29	..
Control	3	0.34	0.078	0.045	.	.	.	.
Cortisone 10 $\times$ 2 mg.	10	0.31	0.058	0.018	0.56	..	0.21	..
Control	8	0.30	0.054	0.019	.	.	.	.
Cortisone 15 $\times$ 2 mg.	8	0.30	0.064	0.023	0.20	..	0.18	..
Control	7	0.22	0.057	0.022	.	.	.	.
Cortisone 5 $\times$ 4 mg.	6	0.18	0.042	0.017	1.42	..	0.29	..
Cortisone 5 $\times$ 8 mg.	7	0.27	0.050	0.019	1.92	..	0.08	..
Control	5	0.38	0.048	0.022	.	.	.	.
DOCA 10 $\times$ 2 mg.	5	0.34	0.047	0.021	1.47	..	0.31	..

*Hydroxyproline concentration of quadriceps femoral muscle.* — The mean hydroxyproline concentration in each cortisone treated group increased. This change was statistically significant in all groups except for those which had been treated with 2 mg. of cortisone during 5 days. Treatment with DOCA did not cause any change in hydroxyproline content of muscle. (Table 17.)

The increase in hydroxyproline content of quadriceps femoral muscle seemed to have some relation to the amount of cortisone used for the treatment of animals (Fig. 5. and 6.).

TABLE 17. *Hydroxyproline concentration in quadriceps femoral muscle.  $\gamma$ /mg. of dry-fat-free tissue.*

Group	Number of animals	Mean conc.	Standard deviation of concentration	Standard error of the mean	Comparison with the control group		Comparison with the regression line	
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
Control	10	2.11	0.70	0.22	.	.	.	.
Cortisone 5 $\times$ 2 mg.	9	2.31	0.63	0.21	0.65	..	1.79	..
Hydrocortisone 10 $\times$ 2 mg.	10	2.47	0.28	0.087	1.51	..	0.91	..
Control	4	3.32	0.43	0.21	.	.	.	.
Cortisone 10 $\times$ 2 mg.	10	5.14	1.24	0.39	4.08	0.01	5.72	0.001
Control	10	2.46	0.69	0.22	.	.	.	.
Cortisone 15 $\times$ 2 mg.	9	4.30	1.20	0.40	4.04	0.001	3.86	0.001
Control	10	3.11	0.57	0.18	.	.	.	.
Cortisone 5 $\times$ 4 mg.	8	3.98	0.60	0.21	3.14	0.01	4.40	0.001
Cortisone 5 $\times$ 8 mg.	9	4.16	0.51	0.17	4.23	0.001	5.73	0.001
Control	6	2.71	0.54	0.22	.	.	.	.
DOCA 10 $\times$ 2 mg.	5	2.79	0.53	0.24	0.25	..	0.54	..

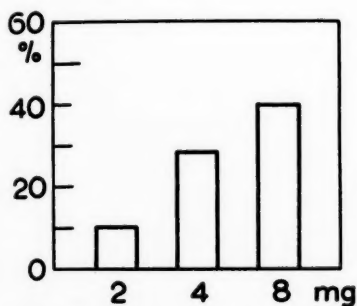


FIG. 5. The increase of mean hydroxyproline concentration of quadriceps femoral muscle in animals treated during 5 days with different daily doses of cortisone, as per cent of corresponding control mean.

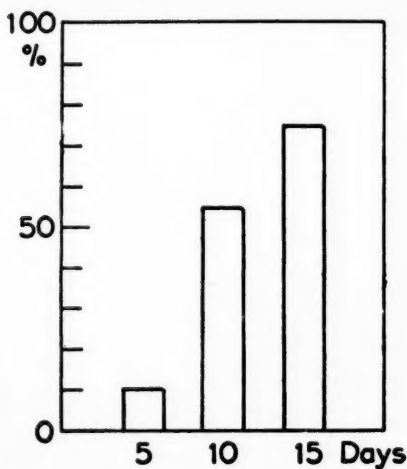


FIG. 6. The increase of mean hydroxyproline concentration of quadriceps femoral muscle in animals treated daily with 2 mg. of cortisone, as per cent of corresponding control mean.

When all the controls in parts I B, II and III of this investigation were grouped together, the following regression equations were obtained

- Hydroxyproline concentration in heart* ( $\gamma$ /mg. wet weight) =  $0.47 - 0.001 \times \text{weight of heart (mg.)}$
- Hydroxyproline concentration in quadriceps femoral muscle* ( $\gamma$ /mg. dry fat-free weight) =  $2.93 - 0.0039 \times \text{weight of muscle (mg.)}$

The lines represented by the equations are shown in Figs. 7 and 9 together with actual individual values in the control group. Deviations of mean values of the experimental groups from the regression lines are shown in Figs. 8 and 10.

These regression equations differ from those given for un-injected controls on page 32. The injected controls also show a wider scatter. The following equation was calculated, by excluding the controls of group B, *i.e.* with the largest scatter:

*Hydroxyproline concentration in quadriceps femoral muscle* ( $\gamma$ /mg. dry fat-free weight) =  $3.68 - 0.0146 \times \text{weight of muscle (mg.)}$ .

However, re-calculation of *t*-tests for comparison of experimental groups with this regression equation revealed that no essential changes took place in results reported in Table 17.

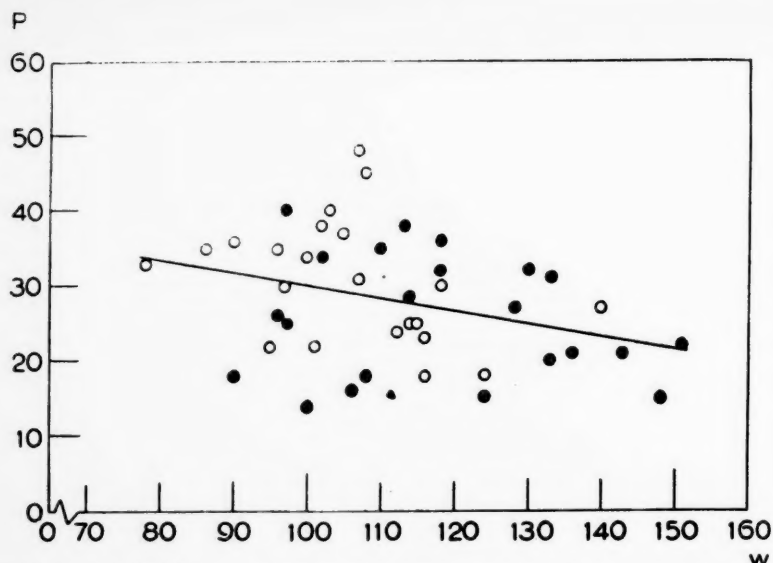


FIG. 7. Individual hydroxyproline concentration and absolute weight of heart in the control groups of Parts I B, II, and III. Circles indicate the individual values of females, and dots those of males. The regression line corresponds to the equation: Hydroxyproline concentration ( $\gamma/\text{mg. wet weight}$ ) =  $0.47 - 0.0017 \times \text{weight of heart}$ .

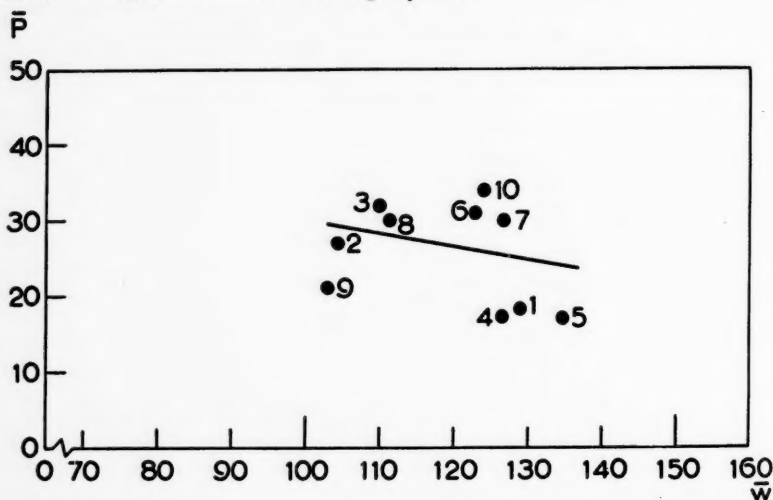


FIG. 8. Deviations of mean hydroxyproline concentration and absolute weight of heart in the groups treated with ACTH or adrenocortical hormones from the regression line of corresponding controls. (Group 1: cortisone 4 mg. daily during 5 days; Group 2: cortisone 8 mg. daily during 5 days; Group 3: ACTH 1 int.unit daily during 5 days; Group 4: cortisone 2 mg. daily during 5 days; Group 5: Hydrocortisone 2 mg. daily during 10 days; Group 6: cortisone 2 mg. daily during 10 days; Group 7: cortisone 2 mg. daily during 15 days; Group 8: adrenalectomy 15 days before; Group 9: ACTH 1 int.unit daily during 10 days; Group 10: DOCA 2 mg. daily during 10 days.)

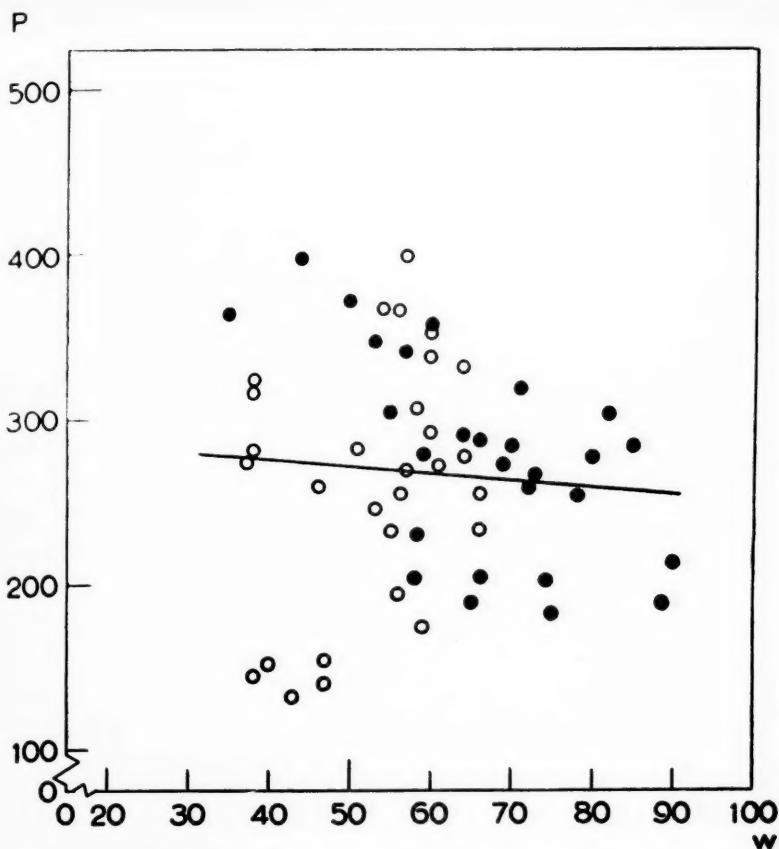


FIG. 9. Individual hydroxyproline concentrations and absolute weights of quadriceps femoral muscles in the control groups of Part I B, II and III (circles—female, dots—male). The regression line corresponds to the equation: hydroxyproline concentration ( $\gamma$ /mg. dry fat-free weight)  $2.93 - 0.0039 \times \text{weight of quadriceps femoral muscle (mg.)}$

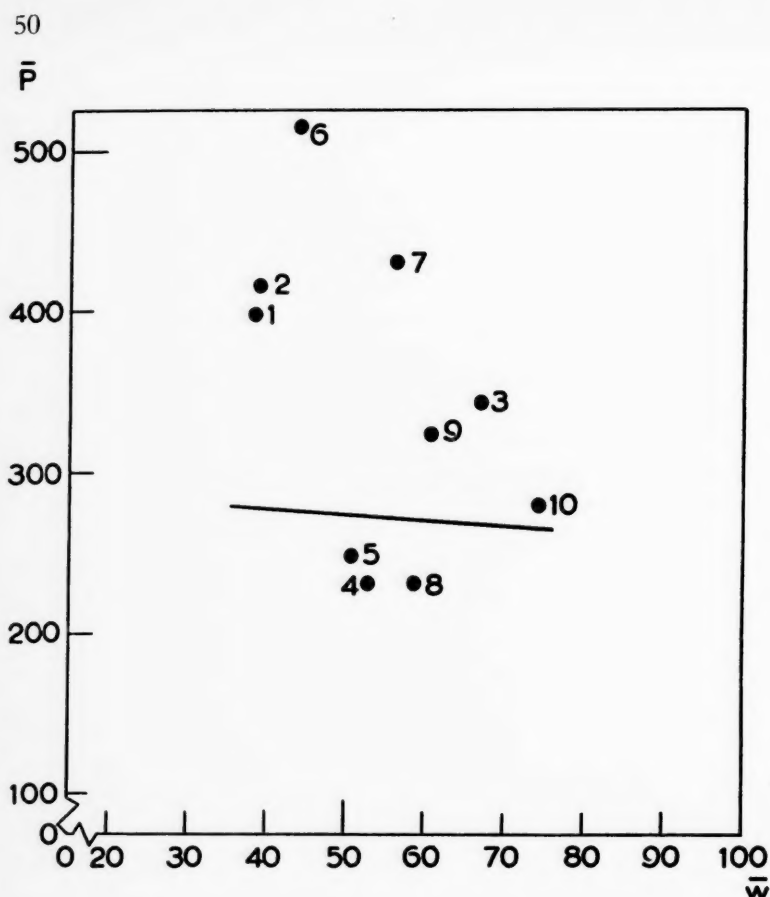


FIG. 10. Deviations of mean hydroxyproline concentration and absolute weight of quadriceps femoral muscle in the groups treated with ACTH or adrenocortical hormones from the regression line of corresponding controls. (The numbering of the groups is the same as in Fig. 8.)

### III. ADRENALECTOMY

*Operation.*—The operation was performed under ether anesthesia. The animal was placed on the operating table in supine position, and the skin of the thoracolumbal region was shaved and cleansed with alcohol. A transverse incision was made at the level of first lumbar spines, and the skin with underlying subcutaneous fat was removed from the lumbodorsal fascia. The longitudinal dorsal muscles were then penetrated on both sides with small pointed scissors by blunt dissection near the vertebral column. The opening was 2 to 3 mm. long. The upper pole of kidney and the adrenal gland were now visible. The gland was grasped with a very small, sharp-pointed forceps and lifted up to the wound. The adrenals were removed one after another with the capsule by squeezing the pedicles with another sharp-pointed forceps. The bleeding was minimal, hardly visible. The opening in muscles was closed with silk-worm suture, one on both sides. The skin was closed with uninterrupted silk-worm suture. The mice tolerated the operation well and recovered quickly. After a few hours they moved about in the cage freely and showed no sign of discomfort. If there was any complication during the operation, or if there was any doubt about the complete removing of both adrenals, the animals was discarded and killed at once. The animals were allowed to drink 1 per cent saline solution instead of water during the period after adrenalectomy. After a period of 15 days, the animals were killed and dissected, as previously described. The chemical analyses were also performed like in previous sections.

Altogether 20 mice were adrenalectomized, and 11 mice were used as controls.

## RESULTS

In adrenalectomized animals no residue of adrenal gland or its capsule and no sign of post-operative bleeding were visible.

*Weight of the animals.* — No significant change took place in the body weight of adrenalectomized mice as compared to the control group. (Table 18.)

TABLE 18. *Weight of the animals, gm.*

Group	Number of animals	Mean weight	Standard deviation of weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	11	20.3	2.8	0.84	.	.
Adrenal-ectomized	20	21.4	2.8	0.62	1.06	..

*Weight of heart and quadriceps femoral muscle.* — The relative weights of heart and quadriceps femoral muscle were unaltered in adrenalectomized group. (Table 19.)

TABLE 19. *Relative weights of heart and quadriceps femoral muscle, mg./100 gm.*

Group	Number of animals	Mean relative weight	Standard deviation of relative weight	Standard error of the mean	Comparison with the control group	
					<i>t</i>	<i>P</i>
Control	9	520	58	19	.	.
Adrenal-ectomized	18	521	79	19	0.03	..
Control	11	262	74	22	.	.
Adrenal-ectomized	19	276	32	7	0.61	..

*Hydroxyproline concentration in heart.* — The hydroxyproline concentration in heart showed no significant change after adrenalectomy. (Table 20.)

TABLE 20. *Hydroxyproline concentration in heart,  $\gamma$ /mg.*

Group	Number of animals	Mean conc.	Standard deviation of conc.	Standard error of the mean	Comparison with the control group		Comparison with the regression line	
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
Control	9	0.34	0.069	0.023	.	.	.	.
Adrenalectomized	18	0.30	0.10	0.025	1.16	..	0.07	..

*Hydroxyproline concentration in quadriceps femoral muscle.* — A significant decrease of hydroxyproline concentration was observed in the adrenalectomized group. (Table 21.)

TABLE 21. *Hydroxyproline concentration in quadriceps femoral muscle,  $\gamma$ /mg.*

Group	Number of animals	Mean conc.	Standard deviation of conc.	Standard error of the mean	Comparison with the control group		Comparison with the regression line	
					<i>t</i>	<i>P</i>	<i>t</i>	<i>P</i>
Control	11	2.90	0.61	0.19	.	.	.	.
Adrenalectomized	19	2.30	0.68	0.16	2.48	0.02	2.20	0.05

## DETERMINATION OF TOTAL NITROGEN

*Method.* — Determinations were performed according to the micro-Kjeldahl technique. Altogether 32 determinations were made, 15 of which from heart and 17 from quadriceps femoral muscle samples.<sup>1)</sup> Before the analysis, animals were treated with ACTH or cortisone injections during 5 days. The amount of hormones had previously proved to produce changes in hydroxyproline concentration.

*Results.* — No change was observed in the total nitrogen concentration of heart or quadriceps femoral muscle of animals treated with ACTH or cortisone.

Swimming 1 hour daily during 5 days and 2 hours daily during the subsequent 5 days did not either cause any change. (Table 22.)

TABLE 22. Total nitrogen concentration of heart and quadriceps femoral muscle,  $N \text{ (mg.)} / \text{fresh weight of organ (mg.)}$ .

	Heart	Muscle
Control .....	0.033	0.032
	0.032	0.033
	0.029	0.036
	0.029	0.036
	..	0.033
ACTH 1 i.u. daily during 5 days	0.034	0.033
	0.034	0.035
	0.031	0.036
Cortisone 4 mg daily during 5 days .....	0.032	0.034
	0.030	0.035
	0.032	0.032
Swimming 1 hour daily during 5 days .....	0.032	0.035
	0.032	0.035
	..	0.032
Swimming 2 hours daily during 5 days .....	0.032	0.030
	0.034	0.035
	0.033	0.035

<sup>1)</sup> Determinations were made by miss Ilona Sinkkonen.

## WEIGHT OF ADRENALS

In the rat the relative weight of the adrenals is greater in the male (Farris and Griffith 1949). The results of the present investigation on mice seemed to be in accordance with this statement. Therefore, the relative weights of both sexes were presented separately (Table 23).

TABLE 23. *Relative weight of adrenals (mg./10gm.)*

Group	Males		Females	
	Mean	Standard error of the mean	Mean	Standard error of the mean
Control	1.48	0.12	2.50	0.10
Swimming 1 h $\times$ 5	2.00	0.24	2.50	0.24
Swimming 1 h $\times$ 10	1.97	0.24	2.72	0.25
Swimming until exh. $\times$ 10	1.80	0.19	2.41	0.19
ACTH 1 i.u. $\times$ 5	1.18	0.14	2.72	0.08
ACTH 1 i.u. $\times$ 10	1.41	0.10	2.65	0.22
Cortisone 2 mg $\times$ 5	0.90	0.05	2.30	0.25
Cortisone 4 mg $\times$ 5	1.08	0.10	2.23	0.14
Cortisone 8 mg $\times$ 5	1.32	0.14	1.82	0.12
Cortisone 2 mg $\times$ 15	0.72	0.19	1.66	0.21
Hydrocortisone 2 mg $\times$ 10	0.92	0.14	3.04	0.15
DOCA 2 mg $\times$ 10			2.60	0.41

By interpreting the results it must be kept in mind that in weighing of adrenals of mice, large errors may have been induced by differences in the amount of tissue removed from the surface of the very small-sized gland.

The present results indicate that the training of mice was insufficient to produce any significant adrenal hypertrophy. Cortisone treatment, however, caused a marked decrease in the relative weight of adrenals. This change is often called »compensatory atrophy» (Selye 1950).

Treatment of animals with ACTH failed to cause any marked change in the weight of adrenals.

## SYNOPSIS OF RESULTS

*Body weight of animals.*

- I. A decrease was observed in all trained groups.  
Treatment with ACTH caused no change.
- II. An increase took place when the animals were treated with 2 mg. of hydrocortisone daily during 5 days.  
Treatment with cortisone or DOCA did not cause any significant change.
- III. No change was observed in adrenalectomized animals 15 days after operation.

*Relative weight of quadriceps femoral muscle.*

- I. An increase took place in the three groups which were most trained by swimming.  
Treatment with ACTH seemed to cause no change.
- II. The group treated with 4 mg. of 8 mg. of cortisone daily showed a decrease. In other cortisone treated groups, and the groups treated with hydrocortisone or DOCA no change took place.
- III. In the adrenalectomized animals no change was observed 15 days after the operation.

*Relative weight of heart.*

- I. In all groups trained by swimming an increase was observed.  
Treatment with ACTH did not cause any change.
- II. An increase was observed in all cortisone treated groups.  
Hydrocortisone treatment had a similar effect. DOCA did not cause any change.
- . Adrenalectomized animals showed no change.

*Hydroxyproline concentration of heart.* — The results obtained concerning the hydroxyproline concentration of heart were rather irregular. The statistically significant changes were:

I. An increase in animals which during 10 days swam until exhaustion. Like in quadriceps femoral muscle, this change was significant when compared with age controls, but not significant when compared with the weight controls (regression line).

An increase after a treatment with 1 i.u. of ACTH during 5 days.

II. An increase after a treatment with a daily dose of 8 mg. of cortisone.

A decrease after a treatment with a daily dose of 2 mg. of cortisone during 5 days.

III. Adrenalectomized animals showed no significant change in the hydroxyproline concentration of heart.

*Hydroxyproline concentration of quadriceps femoral muscle.* — Following statistically significant changes were observed:

I. An increase in animals which during 10 days swam until exhaustion. This change was significant when compared with the age controls, but it was statistically insignificant when compared with the weight controls (regression line).

An increase after a treatment with 1 i.u. of ACTH daily during 5 days.

- II. An increase after a treatment of animals with
  - 2 mg. of cortisone daily during 10 and 15 days,
  - 4 mg. of cortisone daily during 5 days, and
  - 8 mg. of cortisone daily during 5 days.

III. A decrease was observed 15 days after adrenalectomy.

*Total nitrogen concentration of heart and quadriceps femoral muscle.*

No change in total nitrogen concentration was found in the animals trained by swimming. Similarly, the ACTH or cortisone treated animals were without a change.

*Relative weight of adrenals.*

- I. No significant change took place in the weight of adrenals, when the animals were trained by swimming. A slight increase was observed after ACTH treatment.
- II. In cortisone treated groups a decrease of weight of adrenals was observed.

## DISCUSSION

There are only few previous publications concerning the effect of corticoids on hydroxyproline. Roberts *et.al.* (1951) noticed an increase of free hydroxyproline in chick embryos treated with cortisone, and suggested that this was due to a destruction of connective tissue proteins. Ziff *et al.* (1954), however, did not find change in urinary excretion of hydroxyproline in cortisone treated human patients.

In the present investigation, changes observed in hydroxyproline concentration of quadriceps femoral muscle were rather systematical. *Treatment with cortisone constantly caused an increase of hydroxyproline concentration, and a similar result was obtained after a treatment with ACTH.* Muscular exercise, however, failed to produce any significant change in hydroxyproline concentration. Since no adrenal hypertrophy took place in trained animals, it seems unlikely that the exercise was sufficient to produce an adrenal stimulus comparable with a daily dose of 1 int. unit of ACTH.

While, futher, adrenalectomy caused a reaction opposite to that of cortisone or ACTH treatment, it seems likely that *cortisone has an increasing effect on the hydroxyproline concentration of quadriceps femoral muscle.*

The results concerning hydroxyproline concentration of heart were rather irregular though some common features with those concerning hydroxyproline concentration of quadriceps femoral muscle were observed, *e.g. a relation of the hydroxyproline concentration and the weight of the organ* in the control material, and an increase of hydroxyproline concentration in some of the cortisone treated groups.

Because the heart of a mouse is very small, it was impossible to get sufficient amounts of pure myocardium for chemical analysis.

Therefore, the heart ventricles were homogenized as a whole. Although great care was taken for removing of atrioventricular heart valves with the auricles under a magnifying glass, irregular amounts of valvular tissue and papillary muscles may have remained attached to the ventricles. While heart valves and papillary muscles are known to be rich of connective tissue, the irregular presence of them in the heart samples may have caused the large variations of the results in both control and experimental groups.

Cortisone treatment has an inhibitory effect on growth of animals (Ingle 1941). Thus, Harkness and Harkness (1954) suggest »the need for caution in interpreting changes in collagen under conditions causing severe loss of body weight (*e.g.* cortisone treatment).» In the present experiments, however, no significant changes in the body weight took place. Neither was the total nitrogen concentration of heart or quadriceps femoral muscle changed by ACTH or cortisone treatment, or by muscular exercise. Thus, the increase of hydroxyproline concentration after ACTH and cortisone treatment, like the decrease after adrenalectomy, apparently can not be explained by changes in the total protein content of muscular tissue.

Hydroxyproline in muscular organs is in all probability contained in connective tissue proteins. Although no conclusive proof of the chemical homogeneity of collagen has been presented, the hydroxyproline content of organs has often been considered a measure of their collagen content. On the other hand, disagreement still exists concerning the hydroxyproline concentration of reticular fibres which form a large and dense network in the spaces of parenchymal tissues. According to Cramer and Little (1953), reticulín has a high concentration of hydroxyproline, but Gustavson (1949) states that precollagen which often is considered indentical with reticulín, has a low hydroxyproline content. Robertson and Schwartz (1953) suggest that precollagen must have a low hydroxyproline content, if at all present in a scorbutic state.

In interpreting the results of the present investigation it must be kept in mind that there are numerous histological and chemical studies which clearly indicate that cortisone has an antianabolic or catabolic effect on collagen. Therefore, the increase in hydroxyproline content of muscle induced by cortisone treatment, in all need, does not indicate an increase in collagen content. However,

it does not seem quite impossible, that cortisone treatment could have caused changes in the relative amounts of amino acids of collagen. More probable is that the changes took place in reticular tissue. Mature collagen has been stated to be metabolically almost completely inert (Neuberger *et al.* 1951), but as shown by Aterman (1954), changes can be induced by cortisone treatment in the early stages of its development.

It seems to be of interest for further studies that the fibrinoid nodules in rheumatic fever contain less hydroxyproline than the normal tissue (Consden *et al.* 1952). Ziff (1952) found no hydroxyproline in materials extracted from fibrinoid by alkali. While cortisone seems to have a specific effect on the metabolism of hydroxyproline, further studies might be of some significance for investigation of the chemistry of rheumatic diseases and in an analysis of the therapeutic effect of cortisone.

## SUMMARY

- 1) The purpose of the present investigation was to study the possible relation between hydroxyproline content in muscular organs and the activity of adrenal cortex in mice.
- 2) The adrenocortical hormone balance in the experimental animals was altered by following different ways:
  - I. a) muscular exercise,  
b) ACTH treatment,
  - II. a) cortisone treatment,  
b) hydrocortisone treatment,  
c) DOCA treatment,
  - III. adrenalectomy.
- 3) Hydroxyproline concentration in heart and quadriceps femoral muscle was determined according to the method of Neuman and Logan. From some samples also the total nitrogen content was determined according to the micro-Kjeldahl technique. In the present material altogether 291 mice were included, 199 of which as experimental and 92 as control animals.
- 4) *An increase in the hydroxyproline concentration of quadriceps femoral muscle was observed after cortisone or ACTH treatment. As a contrary, a decrease was observed after adrenalectomy.* The changes were statistically significant when compared with both age and weight controls.
- 5) *Hydroxyproline concentrations of heart* were rather variable. However, some similar changes with those observed in quadriceps femoral muscle were obtained when the results were compared with corresponding age controls. Thus, an increase in hydroxyproline concentration was observed after treatment with the largest daily dose of cortisone used in present experiments.

- 6) No changes were observed in the total nitrogen concentration of heart and quadriceps femoral muscle.
- 7) The changes observed here indicate that *cortisone in large doses may increase the hydroxyproline concentration of quadriceps femoral muscle*. It was assumed that the change took place in the amount or chemical composition of young connective tissue elements, *e.g.* reticular fibres.

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## APPENDIX

TABLE 24. *Individual values of body weight, and weight and hydroxyproline concentration of heart and quadriceps femoral muscle.*

Sex	Body weight gm.	Heart		Quadriceps femoral muscle	
		weight mg.	hy. conc. $\gamma$ /mg.	weight mg.	hy. conc. $\gamma$ /mg.
Control					
♀	21,0	116	0,23	53	2,47
♀	19,5	..	..	55	2,33
♀	25,5	124	0,18	58	3,07
♀	17,0	72	0,26	32	4,43
♀	22,0	..	..	52	3,20
♀	20,0	86	0,35	53	3,25
♀	21,5	107	0,31	52	2,76
♀	21,5	100	0,34	52	2,82
♀	19,5	..	..	52	3,18
♀	20,0	91	0,33	45	4,10
♀	21,5	90	0,36	44	3,95
♀	19,5	90	0,31	42	3,53
♀	20,5	94	0,28	44	3,48
♀	19,5	92	0,35	46	4,58
♀	20,5	90	0,37	48	3,08
♀	19,5	..	..	41	2,68
♀	21,0	102	0,16	49	4,24
♀	21,5	101	0,30	50	3,24
♀	20,0	94	0,30	54	2,06
♀	18,0	76	0,38	45	3,26
♀	16,5	71	0,40	40	4,85
♀	17,0	70	0,44	40	3,64
♀	16,5	78	0,36	36	4,15
♀	19,5	91	0,26	48	3,11
♀	18,5	75	0,33	44	2,83
♂	23,5	148	0,15	58	2,04
♂	21,0	106	0,16	55	3,06
♂	27,0	..	..	86	1,24
♂	18,0	83	0,36	43	4,10
♂	18,0	..	..	48	3,13
♂	24,0	104	0,42	71	2,00
Swimming 1 hour daily during 5 days					
♀	18,5	91	0,19	55	3,18
♀	21,5	..	..	64	3,50
♀	23,0	110	0,24	62	2,31

Sex	Body weight gm.	Heart		Quadriceps femoral muscle	
		weight mg.	hy. conc. $\gamma$ /mg.	weight mg.	hy. conc. $\gamma$ /mg.
♀	18,0	93	0,20	52	3,34
♀	18,0	90	0,28	45	3,38
♀	15,5	70	0,53	34	3,08
♀	18,0	78	0,37	36	2,36
♀	17,0	86	0,40	32	3,74
♀	19,5	104	0,29	45	3,35
♀	20,0	89	0,33	47	3,95
♀	17,5	84	0,39	31	2,61
♀	15,0	69	0,52	28	2,84
♀	15,5	79	0,30	31	2,63
♀	15,0	75	0,33	35	2,28
♀	17,0	78	0,40	34	1,99
♂	24,0	127	0,23	56	2,77
♂	18,5	93	0,27	46	2,64
♂	19,0	101	0,13	50	2,60
♂	20,5	107	0,12	55	3,07
♂	21,0	90	0,16	53	3,74
♂	18,0	112	0,38	68	2,50
♂	17,0	121	0,19	64	2,00
♂	11,0	92	0,18	49	1,29
♂	9,0	80	0,19	38	1,72
Swimming 1 hour daily during 10 days					
♀	17,0	88	0,35	44	3,41
♀	19,0	91	0,42	42	4,10
♀	19,0	106	0,26	..	..
♀	17,5	81	0,43	54	2,98
♀	18,0	83	0,41	52	5,70
♀	17,5	85	0,33	42	6,63
♀	12,5	76	0,29	35	4,51
♀	16,5	89	0,31	41	2,74
♀	14,5	99	0,23	48	4,42
♀	17,5	..	..	45	4,48
♀	15,0	61	0,47	34	3,65
♀	18,0	97	0,42	44	2,77
♀	16,5	86	0,24	44	4,02
♀	18,0	90	0,33	42	2,58
♀	18,5	87	0,37	42	3,34

Continued

Continued

Sex	Body weight gm.	Heart		Quadriceps femoral muscle	
		weight mg.	hy. conc. $\gamma$ /mg.	weight mg.	hy. conc. $\gamma$ /mg.
♀	18,0	107	0,15	68	2,52
♀	12,0	..	..	44	2,26
♀	12,0	70	0,17	40	3,05
♀	12,0	80	0,37	40	3,47
♂	22,0	120	0,33	56	2,82
♂	20,0	115	0,30	46	3,51
♂	23,0	107	0,30	..	..
♂	14,0	85	0,15	42	3,12
♂	17,0	..	..	53	3,03
♂	19,0	121	0,17	70	3,10
♂	16,0	116	0,37	64	2,91
Swimming 1 hour daily during 5 days and 2 hour's daily during subsequent 5 days.					
♀	15,0	75	0,31	40	5,18
♀	13,5	71	0,44	40	4,39
♀	15,5	80	0,45	40	3,82
♀	19,0	85	0,51	39	3,90
♀	15,5	71	0,55	40	3,43
♀	15,0	80	0,36	37	4,72
♀	15,5	83	0,40	41	4,53
♀	15,5	91	0,40	42	6,32
♀	12,0	72	0,25	32	4,10
Swimming until exhaustion daily during 10 days					
♀	16,0	95	0,38	64	3,54
♀	17,0	110	0,35	68	3,78
♀	20,0	122	0,29	53	6,11
♀	16,0	94	0,30	58	4,50
♀	12,0	98	0,28	41	9,80
♀	16,0	..	..	60	3,97
♀	15,0	100	0,25	50	4,22
♀	14,0	80	0,26	44	4,85
♂	21,0	..	..	74	3,34
♂	22,0	134	0,22	72	3,12
♂	23,0	130	0,28	..	..
♂	25,0	126	0,21	..	..
♂	20,0	129	0,22	63	2,16

Continued

Sex	Body weight gm.	Heart		Quadriceps femoral muscle	
		weight mg.	hy. conc. $\gamma$ /mg.	weight mg.	hy. conc. $\gamma$ /mg.
♂	21,0	120	0,22	86	3,38
♂	23,0	119	0,24	76	2,02
♂	22,0	135	0,15	78	1,98
♂	20,0	112	0,22	66	3,34
♂	22,0	133	0,26	77	4,14
♂	23,0	127	0,18	74	1,91
Swimming 1 hour daily during 5 days and 2 hour's daily during 5 days after 1 weeks rest period					
♀	20,0	92	0,46	51	3,72
♀	18,5	76	0,28	50	3,55
♀	19,0	89	0,28	47	3,57
♀	16,0	72	0,39	35	4,06
♀	18,5	89	0,27	46	4,15
♀	18,0	79	0,35	43	3,10
♀	17,0	78	0,33	38	4,00
♀	19,5	83	0,36	51	2,92
Control					
♀	22,0	..	..	60	3,39
♀	21,0	..	..	56	2,56
♀	23,0	140	0,27	64	3,32
♀	23,0	115	0,25	66	2,56
♀	19,0	97	0,30	66	2,34
♂	20,5	100	0,14	44	3,97
♂	23,5	123	..	69	2,74
♂	19,5	90	0,18	35	3,64
♂	19,5	108	0,18	50	3,72
♂	24,0	136	0,21	66	2,88
Cortisone 4 mg. $\times$ 5					
♀	19,0	..	..	47	3,46
♀	17,5	115	0,22	33	3,73
♀	19,0	125	0,16	42	3,55
♀	19,0	142	0,11	44	3,93
♂	17,5	..	..	28	4,88
♂	15,5	124	0,21	36	4,80
♂	14,5	96	0,21	32	4,17
♂	23,5	172	0,17	45	3,31

Continued

Sex	Body weight gm.	Heart		Quadriceps femoral muscle	
		weight mg.	hy. conc. $\gamma$ /mg.	weight mg.	hy. conc. $\gamma$ /mg.
Cortisone 8 mg. $\times$ 5					
♀	17,5	87	0,30	26	3,67
♀	16,0	122	0,19	33	4,25
♀	18,0	95	0,35	50	3,87
♀	17,0	94	0,27	36	3,97
♂	17,5	96	0,26	38	3,90
♂	18,0	..	..	46	4,83
♂	14,5	108	0,30	30	3,80
♂	22,0	..	..	54	5,19
♂	16,0	130	0,25	36	4,00
ACTH 1 i.u. $\times$ 5					
♀	19,0	..	..	49	4,10
♀	16,5	96	0,31	52	3,98
♀	22,0	..	..	52	..
♀	18,0	98	0,32	47	3,21
♀	18,5	92	0,34	47	3,03
♂	27,0	..	..	88	3,50
♂	27,5	130	0,30	94	..
♂	26,0	130	0,37	80	2,53
♂	25,5	110	0,30	91	3,85
♂	23,5	114	0,27	69	3,08
Control					
♀	21,0	116	0,18	47	1,54
♀	21,0	114	0,29	40	1,53
♀	23,0	110	0,35	51	2,84
♀	18,5	..	..	47	1,33
♀	20,5	96	0,26	43	1,27
♂	30,0	133	0,20	82	3,05
♂	27,0	124	0,15	74	2,02
♂	28,5	151	0,22	85	2,86
♂	30,0	143	0,21	80	2,77
♂	26,5	..	..	88	1,89
Cortisone 2 mg. $\times$ 5					
♀	21,0	97	0,23	41	1,97
♀	15,0	..	..	36	1,74
♀	20,0	128	0,20	43	1,34
♀	20,0	114	0,21	42	2,24
♀	15,5	112	0,18	37	3,20

Continued

Sex	Body weight gm.	Heart		Quadriceps femoral muscle	
		weight gm.	hy. conc. $\gamma$ /mg	weight mg.	hy. conc. $\gamma$ /mg.
♂	24,5	144	0,19	72	2,78
♂	25,0	151	0,11	74	1,85
♂	28,0	149	0,13	66	..
♂	26,5	..	..	66	2,70
♂	21,5	116	0,11	52	2,97
Hydrocortisone 2 mg. $\times$ 10					
♀	17,0	102	0,19	42	2,79
♀	18,0	..	..	37	2,78
♀	18,5	115	0,16	33	2,70
♀	18,0	113	0,24	42	2,28
♀	18,0	113	0,20	41	2,26
♂	22,0	..	..	53	2,46
♂	25,5	154	0,15	60	1,89
♂	25,0	148	0,15	68	2,49
♂	25,0	152	0,14	68	2,57
♂	24,5	181	0,14	62	2,49
Control					
♀	18,0	90	0,36	54	3,68
♀	17,0	103	0,40	57	2,70
♂	21,5	..	..	57	3,42
♂	24,5	97	0,25	53	3,48
Cortisone 2 mg. $\times$ 10					
♀	18,0	112	0,36	..	..
♀	18,0	143	0,27	40	6,51
♀	15,0	114	0,18	30	4,70
♀	21,5	154	0,35	48	4,47
♀	25,0	170	0,36	62	3,16
♀	16,0	101	0,26	28	5,43
♀	19,0	120	0,32	27	4,71
♂	19,0	88	0,35	36	7,70
♂	22,0	137	0,34	76	5,39
♂	17,5	..	..	52	4,74
♂	15,0	93	0,31	42	4,61
Control					
♀	22,0	..	..	56	3,67
♀	20,5	95	0,22	46	2,60

Continued

Sex	Body weight gm.	Heart		Quadriceps femoral muscle	
		weight mg.	hy. conc. $\gamma$ /mg.	weight mg.	hy. conc. $\gamma$ /mg.
♀	21,0	101	0,22	59	1,74
♀	22,0	105	0,37	61	2,74
♀	16,5	78	0,33	38	1,45
♂	29,0	..	..	78	2,54
♂	28,0	133	0,31	75	1,83
♂	24,0	114	0,30	73	2,68
♂	32,0	130	0,32	90	2,13
♂	22,0	102	0,34	71	3,21
Cortisone 2 mg $\times$ 15					
♀	18,0	..	..	38	4,00
♀	17,0	112	0,38	44	6,70
♀	21,0	101	0,33	60	..
♀	19,0	111	0,23	41	3,70
♀	20,0	120	0,22	48	2,85
♀	17,0	100	0,34	40	3,52
♂	28,0	..	..	90	4,09
♂	23,0	148	0,36	64	4,82
♂	28,0	174	0,26	92	3,46
♂	18,0	150	0,24	46	5,57
Control					
♀	20,0	86	0,35	38	3,25
♀	21,5	107	0,31	37	2,76
♀	21,5	100	0,34	38	2,82
♀	19,5	..	..	38	3,18
♀	17,0	102	0,38	57	4,00
♀	18,5	107	0,48	64	2,80
♀	19,5	112	0,24	60	3,56
♀	17,5	96	0,35	56	1,95
♀	24,0	118	0,30	60	2,92
♂	26,0	128	0,27	59	2,80
♂	18,0	..	..	65	1,91
Adrenalectomy					
♀	22,0	95	0,31	..	..
♀	21,0	70	0,24	47	2,20
♀	24,0	122	0,29	58	2,88
♀	22,0	..	..	57	3,06
♀	19,0	94	0,35	53	2,38
♀	18,0	81	0,28	48	1,02
♀	19,0	88	0,30	42	1,18
♂	25,0	126	0,31	70	1,95

Sex	Body weight gm.	Heart		Quadriceps femoral muscle	
		weight mg.	hy. conc. $\gamma$ /mg.	weight mg.	hy. conc. $\gamma$ /mg.
♂	19,0	..	..	52	1,79
♂	23,5	117	0,22	76	2,63
♂	22,5	111	0,20	62	2,69
♂	21,5	113	0,22	70	2,06
♂	22,5	116	0,67	49	3,16
♂	28,0	150	0,29	80	2,21
♂	23,5	135	0,29	69	1,69
♂	17,0	112	0,17	48	2,46
♂	20,0	112	0,28	60	1,76
♂	21,0	126	0,30	63	2,55
♂	17,0	106	0,31	47	3,80
♂	22,0	138	0,32	70	2,32
Control					
♀	21,0	116	0,23	53	2,47
♀	19,5	..	..	55	2,33
♀	25,5	124	0,18	58	3,07
♂	23,5	148	0,15	58	2,04
♂	21,0	106	0,16	55	3,06
ACTH 1 i.u. $\times$ 10					
♀	18,0	..	..	40	3,07
♀	18,0	74	0,19	44	3,39
♀	21,0	94	0,15	52	..
♀	21,0	90	0,17	52	3,17
♂	25,0	140	0,25	78	3,18
♂	24,0	118	0,29	70	3,38
♂	26,0	..	..	86	3,19
Control					
♂	20,0	118	0,32	72	2,60
♂	20,0	..	..	66	2,05
♂	20,0	108	0,45	64	2,92
♂	19,0	113	0,38	70	2,84
♂	18,0	97	0,40	58	2,26
♂	20,0	118	0,36	60	3,57
DOCA 2 mg. $\times$ 10					
♂	25,0	..	..	86	1,87
♂	25,0	142	0,27	84	3,12
♂	22,0	110	0,37	70	..
♂	24,0	124	0,34	66	2,84
♂	24,0	131	0,32	76	3,12
♂	21,0	114	0,39	64	3,02

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